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DECHLORINATION OF AROMATIC XENOBIOTIC COMPOUNDS BY ANAEROBIC MICROORGANISMS

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The objective of this effort was to determine if anaerobic microorganisms are capable of degrading chlorinated aromatic compounds with nonadjacent unsubstituted carbonatoms. A secondary objective was to define the nutritional requirements for these organisms and extend their substrate range. This project was in response to an unsolicited proposal of the same name. An existing organism which had the capability to dechlorinate benzoate was selected for additional screening against other halogenated aromatics. This isolate was capable of dechlorinating a variety of compounds only in the presence of a supporting consortium of anaerobic organisms. Initial studies using trichloroethylene and 2,3,7,8-tetrachlorodibenzo-p-dioxin as a substrate were not successful. Keywords

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EXECUTIVE SUMMARY

A. OBJECTIVE OF STUDY

The purpose of this research was to assess whether anaerobic dehalogenation occurs on a variety of chlorinated aromatic structures. Special attention was given to aromatic compounds devoid of adjacent nonsubstituted carbon atoms. These structures are often not amenable to aerobic aromatic degradation. The anaerobic dechlorination reaction can remove chlorine atoms, thereby making the ring more susceptible to aerobic or anaerobic degradation. Additional objectives were to understand the mechanism of reductive dehalogenation and to determine the environmental factors that may influence the rate of dehalogenation.

Compounds studied in some detail were the chlorobenzoates and chlorophenols, hexachlorobenzene, and the pesticides, pentachlorophenol, 2,4-D and 2,4,5-T. Studies on the reductive dechlorination of ^{36}Cl- 2,3,7,8-TCDD have been initiated but are incomplete at this time.

The sources of anaerobic dechlorinating bacteria were the pure culture DCB-1 isolated by Shelton and Tiedje (Reference 1) which dechlorinates chlorobenzoate, anaerobic communities from sediments and sludges which have been enriched on chlorobenzoate or chlorophenols, and fresh (unacclimated) anaerobic digestor sludge from municipal wastewater treatment plants. Studies were conducted using a closed incubation system consisting of glass serum bottles sealed with butyl rubber septa. Anaerobic upflow column reactors were also studied for their possible use in treating aqueous waste streams.

B. FINDINGS

In our earlier studies we had enriched a culture from sludge that degraded 3-chlorobenzoate, but we had not been able to enrich this activity from sediments. This was an important deficiency if one wants to enhance cleanup of polluted sediments. Dehalogenating activity successfully enriched was using a lake sediment and 3-Br benzoate as substrate. The activities were eventually enriched to the same specific activity as for the original sludge enrichment. Despite enrichment on the bromo analogue, the sediment enrichment showed a higher rate of dechlorination of 3-Cl benzoate than of 3-Br benzoate. Various methods were used to isolate the active sediment organism but were unsuccessful, including methods which successfully isolated the sludge dechlorinator. The sediment organism was different than the sludge strain (DCB-1) because the DCB-1 morphotype was absent from the sediment enrichment and the DCB-1 isolation methods were unsuccessful. Thus, there must be some diversity among the organisms in nature capable of dechlorination.

The previously isolated dechlorinating strain, DCB-1, was further characterized to understand its physiology. This would aid growth and dechlorination and establish its taxonomic position. We have shown this organism has no known close relatives and has not been previously described. It is a large, nonmotile, gram-negative rod with a unique collar surrounding each cell. The substrate range is extremely limited. Pyruvate was the only

substrate of 53 tested that was able to support reasonable growth. DCB-1 likely can also live as a scavenger because it was isolated on rumen fluid and responded with improved growth when rumen fluid or trypticase supplements were added to the medium. A particularly important contribution to the understanding of DCB-1 was the discovery that this organism showed improved growth when thiosulfate or sulfite were provided as an electron acceptor. The culture cannot, however, be maintained with sulfate as the electron acceptor. The dechlorinator has desulfoviridin and cytochrome c, both typical of the genus Desulfovibrio. Thus, DCB-1 is a type of sulfidogen, but is apparently not closely related to the known members of this genus.

It is not likely that the catalyst in the DCB-1 response for dechlorination has evolved for this purpose because Cl-benzoate has only recently been found in the environment. If we could identify the natural substrate of this "enzyme", we would have a much better assay to use on studies to enhance dechlorination. The only other aromatic substrate that we found metabolized was 3- and 4- methoxybenzoate. The demethoxylation (or demethylation) occurred more rapidly than dechlorination. However, the reaction was not inhibited by sulfite and did not show inhibition by 3-Cl benzoate as a substrate analogue.

Anaerobically digested municipal sewage sludge which had been acclimated to monochlorophenol degradation for more than 2 years was shown to degrade pentachlorophenol (PCP). Di-, tri- and tetrachlorophenols accumulated when PCP was added to the individual acclimated sludges. When the 2-chlorophenol (2-CP), 3-CP, and 4-CP acclimated sludges were mixed in equal volumes, PCP was completely dechlorinated. The same results were obtained in sludge simultaneously acclimated to the three monochlorophenol isomers. With repeated PCP additions, 3,4,5-trichlorophenol, 3,5-dichlorophenol, and 3-CP accumulated in less than stoichiometric amounts. All chlorinated compounds disappeared after PCP additions were stopped. Incubations with ^{14}C-PCP resulted in 66 percent of the added ^{14}C being mineralized to $^{14}CO_2$ and $^{14}CH_4$. Technical grade PCP was initially found to be degraded at a rate very similar to that of reagent grade PCP, but after repeated additions, the technical PCP was degraded more slowly. Pentabromophenol was also rapidly degraded by the mixture of acclimated sludges. These results clearly show the complete reductive dechlorination of PCP by the combined activities of three chlorophenol degrading populations.

Anaerobic upflow bioreactors were used to study the reductive dechlorination of chlorinated phenols. The bioreactors could maintain dechlorinating activity for over 1 year with chlorophenols as the sole carbon source. The chlorophenols were mineralized to methane and carbon dioxide in the bioreactors. Approximately 40 percent of the added chlorophenol was recovered as either methane or carbon dioxide. The substrate range for dechlorinating activity in the bioreactors included meta-chlorophenol, a mixture of ortho-, meta- and parachlorophenols and 3,4,5-trichlorophenol. Chlorophenol loading rates of up to 20 mg per liter reactor per day at a hydraulic retention time of two days were achieved at a substrate conversion efficiency of 95 percent. The bioreactors enriched for three dominant morphological types of bacteria tentatively identified as a

phenol-degrader and two methanogens including Merhanothrix sp. and Methanosarcina sp. The majority of the biomass and dechlorinating activity accumulated in the bottom of the bioreactor in the form of a sludge blanket and was not associated with the glass beads located in the middle of the bioreactor.

Anaerobic biodegradation of 4-chlororesorcinol was studied in fresh sludge and enrichment culture. Complete dechlorination required about 4 weeks and occurred without a lag time. An anaerobic microbial community capable of degrading 4-chlororesorcinol was enriched with 4-chlororesorcinol as a sole carbon energy source in a defined mineral medium. This community consisted of three to four different morphological types. Dechlorination in mineral medium was characterized by a long lag period (4 weeks). Addition of yeast-extract or rumen fluid markedly enhanced the dechlorinating activity. The activity was increased from ca 13 μM to 75 μM per day and the lag period was reduced from 4 weeks to 3 days in presence of either 0.1 percent yeast extract or 5 percent rumen fluid. The study also suggested that the dechlorinating activity is not inhibited by the reaction product -- resorcinol, even at high concentration. On the contrary, enhanced dechlorination was noted in enrichments fed with mixtures of substrate and products.

Hexachlorobenzene (HCB) was dechlorinated in anaerobic digestor sludge from a source that had previously shown the ability to dehalogenate chlorinated phenols. The dechlorination reaction occurred at a much slower rate for HCB as compared to the chlorophenols. In fresh unacclimated sludge, approximately 30 percent of the added HCB was removed over 14 weeks. The dechlorination of HCB was evidenced by the accumulation of 1,3,5-trichlorobenzene which accounted for approximately 40 percent of the HCB disappearance. The same sludge which had been autoclaved showed no HCB degradation.

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PREFACE

This report was prepared for the Air Force Engineering and Services Center, Engineering and Services Laboratory, Tyndall AFB, Florida 32403-6001, under Job Order Number 1900 7023. The principal contractor for this effort was Michigan State University, East Lansing, Michigan. This report covers research conducted between June 1983 to May 1986.

This report has been reviewed by the Public Affairs Office (PAO) and is releasable to the National Technical Information Services. At NTIS it will be available to the general public, including foreign nationals.

This technical report has been reviewed and is approved for publication.

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SECTION I

INTRODUCTION

A. OBJECTIVE

The overall goal of this work was to assess whether anaerobic dehalogenation occurs in a variety of chlorinated aromatic structures for which unsubstituted carbon atoms (if any) are nonadjacent. The chlorobenzoate, chlorophenols, and chlorobenzenes were to serve as model compounds. 2,3,7,8-tetrachlorodibenzo-p-dioxin and trichloroethylene are chemicals of practical interest to the USAF that were also included in studies on evaluating potential for reductive dechlorination.

The proposed work addressed the following six objectives. The results for each objective are detailed in the indicated sections of the report.

1. To examine the dechlorinating ability of the enrichment on chlorobenzoate to metabolize the new model compounds with nonadjacent free carbon atoms. (Section IV)

2. To examine the dechlorinating ability of anaerobic sludge enriched for chlorophenol degradation on the above model compounds. (Sections V, IX)

3. To attempt to obtain additional anaerobic dechlorinating activities for other model compounds. (Sections II, VII, VIII, IX, X)

4. To examine the relationship between chemical structure and dechlorinating activity for the purpose of understanding which substituents limit or enhance the dechlorinating activity. (Sections III, IV)

5. By use of selective pressure, attempt to extend the substrate range of the present dechlorinating organisms to additional chlorinated compounds. (Sections II, VI)

6. To isolate, identify, and characterize the organisms responsible for dechlorination reactions. (Sections III, X)

B. BACKGROUND

It has recently been recognized that anaerobes can involve potentially useful reactions for hazardous waste treatment. Furthermore, anaerobic processes can be more cost-effective than current technology. In our earlier work (References 2, 3), we screened more than 100 different chemicals for biodegradation in anaerobic sludge or eutrophic lake sediments. This survey identified several classes of chemicals that were biodegraded and of particular interest. These included chlorinated aromatic compounds, cresols (References 4, 5), phthalates (Reference 4), and polyethylene glycols (Reference 6). The anaerobic degradation of chlorinated chemicals was of

particular interest because this class has been the most ubiquitous and problematic of the chemical classes that have polluted the environment. Furthermore, the key reaction that we observed -- replacement of the aromatic chlorine(s) with hydrogen -- was a new and particularly promising biotransformation. The removal of only one chlorine will often make the compound more biodegradable and less toxic. Aerobic metabolism of highly chlorinated aromatic chemicals is often restricted because two adjacent ring positions must be free for hydroxylation; the Cl is then removed after ring opening. Thus, the anaerobic dechlorination which occurs before ring opening provides a means to overcome the block preventing aerobic degradation.

Reductive dehalogenation has been known for some time but the previous evidence was for removal of chlorine from nonaromatic carbon atoms This includes two groups of chemicals: the more complex structures often referred to as chlorinated hydrocarbons and the small molecular weight chlorinated solvents. Chemicals in the first class that are susceptible to reductive dechlorination are listed in Table 1, along with the habitats in which that activity has been demonstrated. The activity does not appear to be habitat specific.

The reductive dechlorination of nonaryl Cl in chlorinated hydrocarbons was first reported for DDT in 1967 (Reference 7); however, the next 15 years of research on anaerobic dechlorination to further examples of removal of nonaryl Cl (but not Cl) from the aromatic ring (Table 1). The single exception is the dechlorination of pentachlorophenol (discussed below). Thus, it seems that the aryl-Cl is more difficult to remove than the nonaryl Cl.

The best examples of nonaryl dechlorination are of DDT and lindane (Reference 8). DDT is readily converted to TDE in most if not all anaerobic habitats and by many anaerobically grown microbial cultures. However, dead cells and reduced iron porphyrins are also known to carry out the same conversion. Lindane (BCH or hexachlorocyclohexane) is readily converted to tetrachlorocyclohexane in anaerobic soils and by anaerobic microbial cultures. The most active microorganisms, Clostridium and Citrobacter species, showed nearly completed dechlorination, presumably yielding benzene (Reference 9). These authors noted that only bacteria with iron-sulfur protein dependent H_2 evolution were active and that only substrates whichwere electron donors for ferredoxin-dependent H_2 evolution supported the dechlorination. This dechlorination may also be nonspecific and, perhaps, directly catalyzed by low potential electron donors. Thus, the reason why the nonaryl Cl atoms are more easily dehalogenated may be that they are more easily attacked by the low potential electron carriers found in many microbial cells.

The chlorinated solvents, especially the chlorinated methanes, ethanes, and ethylenes, are important groundwater pollutants for which reductive dechlorination has also recently been shown (References 10, 11, 12, 13). Compounds which were reductively dechlorinated in methanogenic communities from sewage sludge and in anaerobic groundwaters include carbon tetrachloride,

TABLE 1. ANAEROBIC MICROBIAL HABITATS THAT HAVE SHOWN REDUCTIVE DECHLORINATION OF Cl FROM NONAROMATIC C-Cl BONDS (SUMMARIZED FROM REVIEWS OF ESSAC AND MATSUMURA, 1979 AND SETHUNATHAN, 1983).

Chemical	Active anaerobic habitats[a]
DDT	Soil, rumen fluid, sewage sludge, sediments, microbial cultures
Lindane	Soil, sediments, microbial cultures
Toxaphene	Rumen fluid, sediment
Heptachlor	Soil, microbial cultures
Mirex	Sewage sludge
Endrin	Soil
Methoxychlor	Soil

[a]Soil used was made anaerobic by flooding, adding an anaerobic atmosphere and/or adding readily degradable organic matter. Some of the soils were rice paddy soils.

chloroform, tetrachloroethane, tetrachloroethylene, trichloroethane and trichloroethylene. Reduced iron porphyrins were also shown to dechlorinate some of these compounds as well (Reference 14).

Most of our earlier work focused on chlorobenzoates as model compounds. However, chlorobenzoates occur at hazardous waste sites, are a product of certain pollutants, e.g., PCBs, and have some congeners that are herbicides: Amiben (3-amino-2,5-dichlorobenzoic acid), Banvel (dicamba or 2-methoxy-3,6-dichlorobenzoic acid), and TBA (2,3,6-trichlorobenzoic acid).

Two independent observers collaborated our findings of reductive dehalogenation of an aromatic compound by an anaerobic community. Horowitz used eutrophic lake sediments to study the fate of selected chemicals and observed that 4-amino-3,5-dichlorobenzoate was converted to 4-amino-3-chlorobenzoate (Reference 15). At the same time, Shelton was studying compounds that were converted to gas by anaerobic sludge and noted that two of the nine sludges fed 3-chlorobenzoate showed 85 percent of the substrate carbon converted to CH_4 + CO_2 (Reference 3). This enrichment was later shown to produce benzoate as an intermediate (Reference 16) and, therefore, provided the evidence that this community also replaced the aryl Cl with H. Oxygen strongly inhibited the dechlorination in both communities.

The range of halogenated benzoate substrates dehalogenated by the sediment and sludge communities is summarized in Table 2. Without exception

all chlorines in the meta position were removed and none were removed from other positions. For the bromo and iodo substituents the specificity is not apparent; these halogens were removed from the ortho and para positions as well as the meta position. Thus, considerable specificity is shown by these chlorobenzoate dehalogenating communities for the meta chlorine. The number of chlorine substitutions does not seem to be important to the dechlorination because mono, di, and tri chlorinated substrates all yielded to dechlorination.

The dechlorination of chlorobenzoates in sediments was characterized by a particularly lengthy lag period prior to the onset of rapid dechlorination. This period ranged from 3 weeks to more than 6 months depending on chemical (References 15, 17). Because this lag period was more easily studied in sediment, this environment was used to characterize certain aspects of the dehalogenation. The lag period was followed by rapid dechlorination which occurred both for substrates that were completely metabolized to CH_4 + CO_2 as well as for those that showed only a single dechlorination. In the latter case no substrate carbon was available for growth, thus, the lag period cannot be due to a population growth response. Once the acclimation occurred, subsequent dechlorination commenced without lag following the addition of new substrate.

The fact that dechlorination did not occur during this lengthy lag period is also strong evidence that the dechlorination is not a generalized chemical reaction perhaps caused by low potential electron donors such as occurred for the nonaryl dechlorinations described earlier. These donors should be just as prevalent if not more so early in the incubation (lag period) as well as later. There is also evidence that this acclimation was chemical specific. Sediments acclimated to either benzoate, 3-iodo-benzoate or 4-amino-3,5-dichlorobenzoate resulted in an acclimation which degraded that or related substrates but not substrates from the other two groups (Reference 15). Obviously, there is a specific response elicited by the chlorobenzoate that eventually selects for some biochemical capacity to carry out the dehalogenation. In our studies, sediments sterilized by autoclaving, gamma irradiation, or formaldehyde did not show dechlorination (Reference 15).

The chlorobenzoate dechlorinating activity in sludge was studied primarily after enrichment (Reference 18). This enrichment was obtained by repeated additions of 3-chlorobenzoate as the only carbon source and by periodic transfer until a stable community existed, free from the original sludge matrix. This enriched consortium showed dehalogenation rates that ranged from 0.31 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$ for 3-chlorobenozoate to 0.04 $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$ for 4-amino-3,5-dichlorobenzoate (Reference 16). The inhibition of dechlorination of the monochlorosubstrate by dichlorobenzoate was also observed (Reference 18). The dichlorosubstrate, probably acting as a substrate analog, caused a stoichiometric accumulation of the monochloro product. When the dichlorosubstrate was depleted, the inhibition was relieved, and the monochloro product was also degraded. The kinetic pattern of the accumulation and disappearance of these substrates and products showed the phenomenon to be competitive inhibition. Examples of the first substrate in the pathway being the competitive inhibitor of a second reaction in the pathway are rare because this mechanism would prevent growth on such

TABLE 2. SPECIFICITY OF FRESHWATER SEDIMENTS, SLUDGE ENRICHMENT AND DEHALOGENATING STRAIN (DCB-1) FOR POSITION OF HALOGEN FOR DEHALOGENATION (DATA SUMMARIZED FROM SULFITA ET AL., 1982 AND LINKFIELD. 1983).

Substrate DCB-1	Position of halogen removal[a] Sediments	Sludge enrichment	Pure culture
Monohalogen			
2- or 4-Iodobenzoate	2-,4-	2-,4-	nt
3-Iodobenzoate	3	3	3
2- or 4-Bromobenzoate	2-,4-	2-,4-	nt
3-Bromobenzoate	3	3	3
2- or 4-Chlorobenzoate	-	-	-
3-Chlorobenzoate	3	3	3
3- or 4-Fluorobenzoate	-	-	-
2-Fluorobenzoate	2	?	nt
4-Amino-3-chlorobenzoate	-	-	-
Dihalogen			
3,5-Dichlorobenzoate	3,5	3,5	3.5
3,4.Dichlorobenzoate	3	3	3
2,5-Dichlorobenzoate	5	5	5
2,4-Dichlorobenzoate	-	-	-
2,6-Dichlorobenzoate	-	-	-
5-Bromo-2-chlorobenzoate	nt	nt	5
4-Amino-3,5-dichlorobenzoate	5	3,5	5
Trihalogen			
2,3,6-Trichlorobenzoate	3	3	3

[a]/nt = not tested; - = no activity

substrates. However, with mixtures of chemicals existing in waste sites such a phenomenon might be likely. If present, it would result in the inhibition of degradation even if the biochemical pathway existed in the indigenous community.

The sludge community that carried out the dechlorination has been characterized (Reference 1). Eight different organisms were isolated and a ninth, Methanochrix, can be easily recognized by its morphology. These organisms are illustrated in Figure 1 and are placed according to their likely position in the path of carbon flow. Four of these organisms are thought not to play a central role in carbon flow but are likely present as scavengers. These are two anaerobic butyrate oxidizers and the two sulfidogens. The remaining five organisms are thought to have major roles in the carbon and hydrogen flow. At least four are necessary to convert chlorobenzoate to CH_4 + CO_2: (1) the dechlorinating strain, DCB-1, that produces benzoate; (2) the benzoate oxidizing strain, BZ-2 (likely Syntrophus buswelli), that produces H_2 plus acetate, (3) two H_2-consuming methanogens, strains of Methanospirillum and Methanobacterium; hydrogen consumers are necessary to provide thermodynamically favorable conditions for anaerobic benzoate oxidation to proceed; and (4) the acetoclastic methanogen, Methanothrix, which cleaves acetate into CO_2 and CH_4.

One objective of this research was the further characterization of the dechlorinating bacterium because this organism's activity was unique. We have also studied the fate of PCP and other chlorophenols in anaerobic sewage sludges and have shown these compounds to be extensively dechlorinated. In fresh (unacclimated) sludge from primary anaerobic digestors (Jackson, Michigan), the ortho-positions of PCP (chlorine in the 2 and 6 positions) were rapidly dechlorinated to 3,4,5-trichlorophenol, which was subsequently converted to 3,5-dichlorophenol (Reference 19). Monochlorophenol isomers were not observed. 2,4,6-Trichlorophenol followed this same pattern of ortho dechlorination, yielding 4-chlorophenol as the final degradation product. The degradation of PCP by two other anaerobic sludges was considerably slower; whereas the Jackson sludge completely degraded 50 μM PCP within 14 days, only partial PCP degradation was observed after 70 days in the other two sludges (Reference 19). Extensive PCP biodegradation in anaerobic sludge was also observed by Guthrie et al. (Reference 20). PCP inhibited methanogenesis at a concentration of 200 $\mu g\ l^{-1}$ in fresh sludge and at 600 $\mu g\ l^{-1}$ in acclimated sludge.

Reductive dechlorination reactions have also been observed for the mono- and dichlorophenols in fresh and acclimated anaerobic digestor sludge. The monochlorophenol dechlorination was first observed for 2-chlorophenol (Reference 5). Complete conversion of 30 ppm 2-chlorophenol to phenol required approximately 3 weeks in fresh 10 percent anaerobic sludge. All three monochlorophenols inhibited methane productior. In fresh whole sludge, the relative rates of disappearance were: 2-chlorophenol >> 3-chlorophenol > 4-chlorophenol (Reference 21). For dichlorophenols in unacclimated sludge, reductive dechlorination of the Cl group ortho to phenolic OH was observed for each dichlorophenol isomer with such a Cl substituent. 3,4-Dichlorophenol and 3,5-dichlorophenol were persistent during the 6-week incubation (Reference 21).

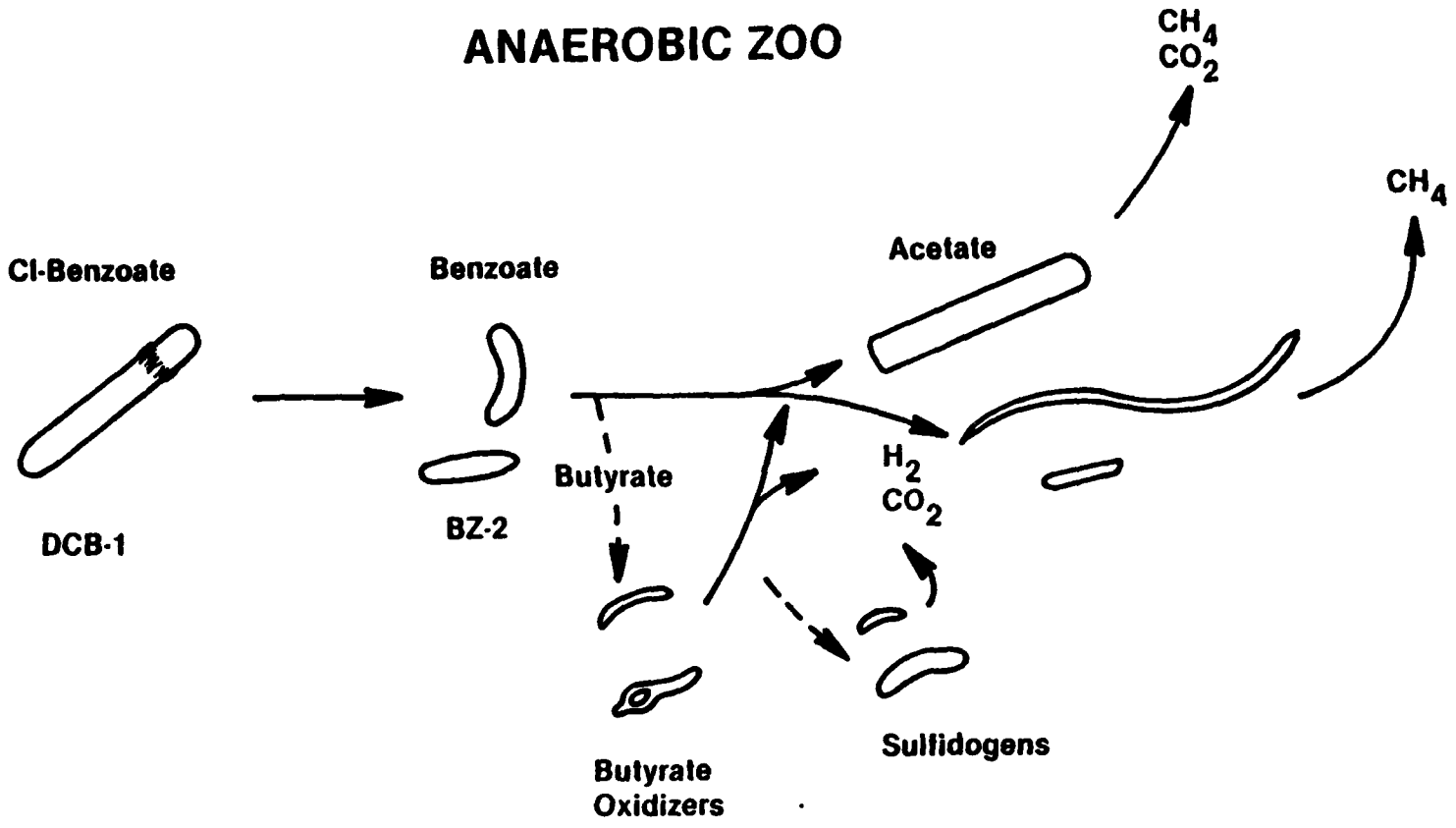

Figure 1. Diagram of the Bacteria Present in the 3-Chlorobenzoate Degrading Consortium. They Are Arranged According to Their Projected Position in the Food Chains.

The same sludge acclimated to either 2-,3-, or 4-chlorophenol gave patterns of degradation distinctly different from those of fresh sludge (Reference 21). For example, sludge acclimated to the degradation of 2-chlorophenol degraded 2- and 4-chlorophenol at equal rates whereas 3-chlorophenol was not degraded. Sludge acclimated to 3-chlorophenol also degraded 4-chlorophenol but not 2-chlorophenol. The rates of degradation were enhanced in the acclimated sludges and previously persistent compounds, 3,4- and 3,5-dichlorophenol, were degraded quite rapidly. At the same time, the ortho-dechlorination of the dichlorophenols was less rapid.

These studies have shown that the outstanding feature of chlorophenol degradation in fresh sludge was the reductive dechlorination of Cl ortho to phenolic OH. This dechlorination could result from enzymatic activity of specific organism(s) or from a more nonspecific process mediated by biologically produced electron carriers. At least two distinct chlorophenol-degrading populations were observed in sludges acclimated to 2- or 3-chlorophenol. These sludges were simultaneously acclimated to the degradation of other chlorophenols. The specificity of the cross-acclimation patterns, and the enhanced rates of degradation, argue for a specific biological mechanism, presumably enzymatic.

The above work used only polar chlorinated aromatic compounds as substrates. Many of the major problem compounds are nonpolar, however, e.g. TCDD, PCBs and chlorinated benzenes. One objective of the research under this contract was to determine whether highly chlorinated nonpolar compounds could also be dechlorinated by anaerobic communities. Some evidence that suggests that we might find such compounds degraded comes from congener profile analysis of sediments. Bailey (Reference 22) has pointed out that dehalogenation of HCB in anaerobic sediments can be inferred from data by Oliver and Nicol (Reference 23) on the distribution of chlorobenzenes in the Great Lakes. Analyses of different HCB congeners in sediment cores revealed that in the older layers the ratio of dichlorobenzene (DCB) and TCBs to HCB and pentachlorobenzene (QCB) increased dramatically. For example, the ratio of 1,4-DCB to HCB increased as follows: 0.41 (0.1 cm; 1976-1980), 0.26 (1-2 cm; 1971-1976), 0.40 (2-3 cm; 1965-1971), 1.44 (3,4 cm; 1958-1965), 1.16 (4,5 cm; 1950-1958), 1.81 (5-6 cm; 1941-1950) and 20 (6,7 cm; 1932-1941). The pattern suggested a slow dehalogenation of HCB in the anaerobic sediment. HCB was to be used as a model compound in this study to see if its anaerobic dechlorination could be more directly established.

A study of PCB congener distribution in upper Hudson River sediments suggested that PCBs were also being reductively dechlorinated by natural populations of anaerobic bacteria (Reference 24). Sediments containing relatively high levels of PCBs (> 50 ppm) all showed losses of up to one-third of the chlorine originally present. This resulted in distinct changes in congener distribution with the less chlorinated PCB congeners becoming relatively more prevalent. Several different patterns of dechlorination were observed at different sites. The high levels of individual mono-, di-, and tri-chlorobiphenyls in these samples could not be explained by simple physical partitioning of the original discharges. Apparently the reductive dechlorination of PCBs occurred in a stepwise fashion until lower chlorinated

congeners were produced which were more difficult to reduce, due to their increasing reduction potentials. This result was consistent with those described earlier for the chlorophenols. The PCB dechlorinations were suggested to be biologically based on the observation of congener selectivity, similar to that observed for the dechlorination of chlorobenzoates (Reference 16).

The intestinal contents of rats have been reported to convert TCB to mono- and dichlorobenzene (Reference 25). In a subsequent study, 12 bacterial strains were isolated and tested for their ability to convert 1,2,4-TCB to 1,2-DCB (Reference 26). Although 1,2-DCB was detected in each of these 12 strains, the percent conversion of TCB was very low (ca. < 1 percent). The dechlorinating activity was highest in Staphylococcus epidermidis (Strain 1) and proceeded only under an atmosphere of H_2. The dechlorinating activity of Strain A was apparently stimulated by NADPH, however, the stimulatory effect was observed for dry, intact, and broken cells.

Reductive dechlorination has been observed for several pesticides in anaerobic sediments, soils and sludges. One of the earliest was the anaerobic dechlorination of techlofthalam (N-(2,3-dichlorophenyl)-3,4,5,6-tetrachlorophthalmic acid) in paddy soil (Reference 27). Two or more monochlorinated products were observed after 2 weeks incubation.

Enrichment cultures obtained from pond sediment completely degraded the herbicide diuron [(3-(3,4-dichlorophenyl-1,1) dimethylurea C)] in 17-25 days. The mode of degradation was removal of the 4-Cl substituent giving 3-(3-chlorophenyl)-1,1-dimethylurea in stoichiometric amounts (Attaway et al., 1982a, 1982b). The monochloro derivative was not degraded.

The herbicide benthiocarb (thiobencarb, S-4-chlorobenzyl N,N-diethylthiocarbamate) used widely in rice fields has been observed to undergo reductive dechlorination in certain flooded soils. The dechlorinated product, S-benzyl N,N-diethylthiocarbamate, appeared to cause dwarfing of rice plants in some paddy fields (Reference 28). Of 17 soils examined, the dechlorination occurred in two soils. Organic amendments to these soils were required for dechlorination. It was suggested that the dechlorinating activity in soil required free solution phosphate and was carried out by facultative anaerobic microorganisms.

In a related study, the reductive dechlorination of CNP (chloronitrofen, 4-nitrophenyl 2,4,6-trichlorophenyl ether) was compared with benthiocarb (Reference 29). The primary dechlorinated product from CNP was 4-aminophenyl 2,6-dichlorophenyl ether which resulted from removal of the para Cl. Subsequent removal of Cl in the 2 and 6 positions occurred to lesser extents. The dechlorination of CNP in soil was slower than that of benthiocarb.

Anaerobic degradation of the pesticides 2,4-D (2,4-dichlorophenoxy acetic acid) and 2,4,5-T (2,4,5-trichlorophenoxy acetic acid) in sewage sludge has been observed (Reference 19). The ether bonds of 2,4-D and 2,4,5-T were rapidly cleaved, giving 2,4-dichlorophenol and 2,4,5-trichlorophenol. This reaction was followed by rapid removal of Cl groups in the ortho positions of

2,4-dichlorophenol and 2,4,5-trichlorophenol. 4-Chlorophenol, released from 2,4-dichlorophenol, was degraded slowly, and 3,4-dichlorophenol, released from 2,4,5-trichlorophenol, was partially dechlorinated to 4-chlorophenol.

The above review work suggests that reductive dechlorination of at least some aromatic compounds occurs but has not been established for several of the more toxic chlorinated pollutants; e.g., PCBs, HCB and dioxins, nor are the conditions favoring or inhibiting these reactions understood. The metabolism of pentachlorophenol and hexachlorobenzene must dramatically illustrate that fully substituted aromatic rings can be dehalogenated. Because the dechlorinating activities tend to show high substrate specificity, it was necessary to put more emphasis on objective 3, to find additional dechlorinating activities. We were successful in finding activities for chlorinated dihydrobenzenes; e.g., Cl-resorcinol, Cl-catechol and Cl-hydroquinone; for the chlorinated benzenes; and for tetrachloroethylene (PCE). At the end of technical efforts, there was no evidence that TCDD and trichloroethylene (TCE) were dechlorinated. We were able to enrich for and characterize some unique organisms involved in dechlorination and did discover that oxidized sulfur compounds inhibit dechlorination. This is of particular importance when marine waters are associated with contaminated sediments.

SECTION II

DEHALOGENATION IN WINTERGREEN LAKE SEDIMENT AND THE ENRICHMENT FOR DEBROMINATING ACTIVITY ON 3-BROMOBENZOATE

A. RATIONALE AND APPROACH

Further knowledge of dehalogenating organisms can only occur through the enrichment of the activity and the isolation of the pertinent organisms. Enrichment for the activity involves gradually switching via several transfers from a sediment or sludge sample to a defined medium where the organic compound undergoing dehalogenation serves as the sole carbon source for the enriched community. Dehalogenation activity on chlorinated benzoates was enriched from anaerobic sludges by this approach (Reference 3).

Enrichments from sediments were not previously achieved and sediment activities were more versatile and diverse than for sludges (Reference 17), Wintergreen Lake sediments were used in an effort to enrich for additional halobenzoate dehalogenating populations. Because the sediments rapidly dehalogenated 3-bromobenzoate, this compound served as the sole carbon source for the attempt. Other objectives were to isolate new dehalogenating species, and to describe the dehalogenation of compounds other than benzoates. This section describes the attempted isolation techniques, and the capacity of Wintergreen Lake sediments to dehalogenate compounds other than the benzoates.

B. MATERIALS AND METHODS

1. Sediment Studies

Anaerobic Wintergreen Lake sediments were sampled, transferred to serum bottles, incubated, and analyzed as described in by Shelton and Tiedje (Reference 3). Sediments were incubated under 100 percent N_2 whereas enrichment media were under 80 percent N_2:20 percent CO_2. Hungate gas probes were used for all media preparations and transfers. The sediments were assayed for methane production prior to the addition of any halogenated compounds. The substrates tested were 2-Cl-, 3-Cl-, 4-Cl., 2,3-diCl-, 3,4-diCl- and 2,3,4-trichlorophenol, 2,4-D and 2,4,5-T. Substrate concentrations were between 10 and 35 ppm. All incubations were at 20°C in the dark. Tests were also performed at 7° and 50-67°C, with 800 µM concentrations of 3-F-, 3-Cl-, 3-Br-, and 3,5-dichlorobenzoate. Stock solutions were prepared in water or as Na^+ salts. Substrates were quantitated with a Waters Model 441 HPLC on a Waters Radial Pak 8C18 column, 10 um particle size, contained in a RCM-100 cartridge holder. The mobile phase was 45 percent acetonitrile and 55 percent water acidified with 5 percent acetic acid. The detection wavelength was 280 nm.

2. Enrichment Techniques

Sediments from the lag time studies (Reference 17) were maintained at 20°C and continually fed 3-bromobenzoate to enrich for the dehalogenating

activity. After consuming approximately 12 mmoles of 3-bromobenzoate, these sediments were transferred without prior dilution to 160 mL serum bottles and immediately diluted 50 percent with mineral salts medium (Reference 3) modified by the addition of 1 mL of 1 mM $NiCl_2 \cdot 6H_2O$ (Reference 30). The unmodified medium served as the basis of all subsequent media used. When the contents of each 160 mL bottle had degraded approximately 6 mmoles of 3-bromobenzoate, further dilution and transfer was accomplished by 50 percent transfer into new modified medium. The frequency of transfer was determined by analyzing the enriched sediments by HPLC for substrate disappearance and the headspace gas by GC for methane production.

This process was continued until the enrichments were calculated to contain only 1 percent of the original sediment. At this time the activity dropped precipitously. In an attempt to recover activity yeast extract (0.01 percent) and glass beads (20 percent by weight; 1.00-1.05 mm diameter; Glasperlen) were added and the extra $NiCl_2 \cdot 6H_2O$ was omitted; this was successful. When the enrichment reached a stable condition utilizing 3-bromobenzoate as sole carbon source, the contents of the serum bottles were transferred to a 1000 mL Erlenmeyer flask maintained in an anaerobic condition by wiring a modified butyl rubber stopper to the top. The modification included the insertion through the stopper of an anaerobic culture tube (Bellco-Glass, Inc.) previously cut in half. This culture tube was then capped by a butyl rubber stopper in a manner that allowed for injecting desired components into the flask. The medium used was the unmodified salt medium with the corresponding quantity of glass beads.

The enrichment was then studied to determine which halogenated aromatic compounds would be dehalogenated or would support the enriched consortium. The compounds were the three isomers of the fluoro, chloro, bromo and iodo monohalogenated benzoates; 2-Cl- and 4-chlorophenoxyacetic acid; 2-Cl-, 3-Cl-, and 4-chlorophenol; phenol; phenoxyacetic acid and phenylacetic acid. Initially the compounds were tested at 20°C and 37°C but due to the improved activity at 37°C this temperature was used for comparison to the sludge enrichment data.

The HPLC was either the same as above or a Varian 5000 coupled to a Hitachi 100-40 spectrophotometer with an attached Altex® 155-00 spectrophotometer flow cell. The column was a Hibar LiChrosorb®, 10 m particle size, RP-18. The mobile phase was a 70:40:13 (H_2O:CH_3OH:CH_3COOH) mixture diluted with methanol. The detection wavelength varied depending upon the substrate used. Methane was monitored on a Carle 8500 microthermistor detector gas chromatograph equipped with a Porapak® QS packed 2 meter column.

3. Isolation Techniques

Several techniques were utilized in an effort to isolate a dehalogenating organism from the enrichment described above. Several carbon sources and substrates including glucose, pyruvate, trypticase, tryptone, yeast extract, pectin, lactate, rumen fluid and humic and fulvic acids extracted (Reference 31) from Wintergreen Lake sediment were used in roll tubes (Reference 1). The enrichment was also added to similar roll tubes in

coculture with the methanogen PM-1 (Reference 1) and the sulfidogen PS-1 (Reference 1) to test whether low H_2 concentrations would be beneficial.

C. RESULTS AND DISCUSSION

Wintergreen Lake sediments have been challenged by myriad halogenated compounds and the expressed activity was described as being confined to the halogenated benzoates (Reference 2). However, we found that these same sediments dehalogenated low concentrations of the selected haloaromatics (Table 3). The dechlorination of phenols was confined to the removal of the ortho chlorine. Phenol, the product of dechlorination, was recovered in stoichiometric quantities (Figure 2) suggesting that an enriched phenol degrading population was not responsible for the dechlorination. The disappearance of 2,4,5-T and the appearance of a product peak resembling 2,5-D suggests that this phenoxyacetic acid can also be dehalogenated.

In these experiments 3-bromobenzoate served as the sole carbon source for the formation of a successful enrichment of debrominating activity from sediment. Although the temperature of these sediments in situ ranges from 4° to 16°C annually, the enrichment was tested at 20° and 37°C to determine if metabolic activity would be increased by an increase in temperature. Because the activity was consistently greater at 37°C (Table 4), this higher temperature was used in further experiments. Rate measurements at 37°C also allowed for a more direct comparison to dehalogenation rate data from previous sludge enrichment data (Table 5). The major improvements in substrate range in the sediment enrichment when compared to the sludge activity were the utilization of 2-F-, 4-Br-, 3-I- and 4-I-benzoate, whereas the remaining benzoate isomers were degraded at approximately the same rate in either enrichment. Halobenzoates were the only compounds that served as carbon sources for the sediment consortium.

In an attempt to isolate the dehalogenating species, the enrichment was diluted and transferred to roll tubes (Reference 1) in medium containing mineral salts and various defined and undefined organic sources. All supported the growth of individual colonies. However, none of the bacterial strains exhibited the ability to dehalogenate. When the enrichment was transferred to roll tubes containing basic salts, 20 percent rumen fluid, and 0.2 percent cellulose, several colony types developed. These were "picked" by Pasteur pipet (Reference 1) and transferred to liquid medium containing basal salts, 20 percent rumen fluid and 0.2 percent cellulose.

The resulting culture dehalogenated 3-bromobenzoate and accumulated benzoate stoichiometrically and did not produce methane. Subsamples of these cultures were diluted and transferred to roll tubes containing 20 percent rumen fluid and 0.2 percent pyruvate. Colonies were picked and transferred to an identical liquid culture medium. This culture contained more than one cell type, but it did dehalogenate 3-bromobenzoate to benzoate, with no further use of the ring. Repeated efforts to purify this culture by roll tubes and isolate a dehalogenating monoculture failed. Several isolates were purified but none was capable of debromination.

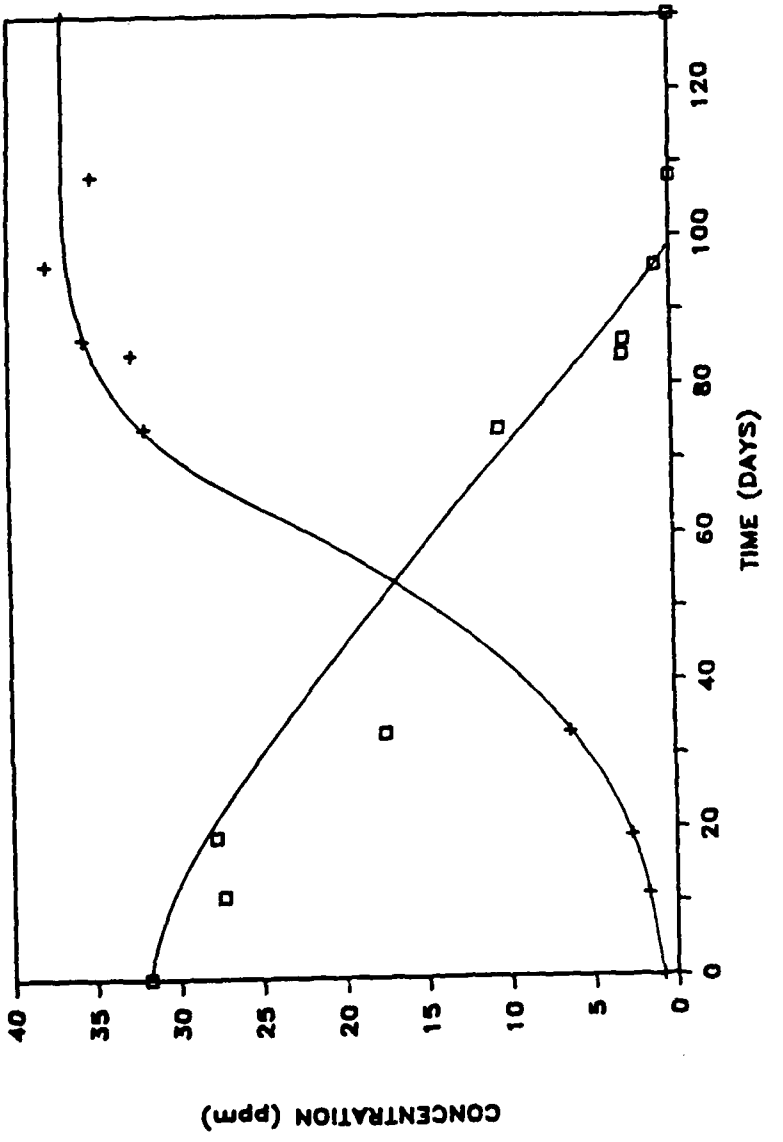

Figure 2. Dehalogenation of 2-Chlorophenol in Wintergreen Lake Sediment.

TABLE 3. ANAEROBIC DEHALOGENATION OF COMPOUNDS OTHER THAN BENZOATES IN WINTERGREEN LAKE SEDIMENTS.

Substrates	Product
	Dehalogenation observed[a]
2-Cl-phenol	Phenol
2,3-di-Cl-phenol	3-Cl-phenol
2,4,5-T	(2,5-diCl-phenoxyacetate)[b]
	No dehalogenation
3-Cl-phenol	---
4-Cl-phenol	---
3,4-diCl-phenol	---
2,3,6-triCl-phenol	
2,4-D	

[a]Samples were incubated up to 150 days.
[b]Product was not 2,4-dichlorophenoxyacetate. The most likely product with the retention time obtained is 2,5-D.

TABLE 4. HEADSPACE METHANE PRODUCED FROM VARIOUS MONOSUBSTITUTED BENZOATES BY THE SEDIMENT/ENRICHMENT INCUBATED AT 20°C AND 37°C.

	Methane produced (mL/l)[a]	
Benzoate substrate	20°C	37°C
2-F-	36.2	46.4
3-Cl-	28.7	46.9
2-Br-	21.9	40.9
3-Br-	42.2	47.0
4-Br-	39.6	36.8
2-I-	39.6	44.7
3-I-	42.4	47.4
4-I-	42.6	47.6

[a]Total CH_4 at 21 days released from 600 μM substrate.

Further attempts to purify the dehalogenating organism involved diluting the above 20 percent rumen fluid -- 0.2 percent pyruvate medium and rolling it into roll tubes containing 20 percent rumen fluid, 0.2 percent pyruvate and 5-10 mM $Na_2S_2O_3$. The resulting colonies were picked, returned to identical liquid medium and grown. These were transferred to 20 percent rumen fluid and 0.2 percent pyruvate and checked for a dehalogenating capability. All isolates failed to express the desired activity.

The realization that reductive dehalogenation occurs anaerobically (References 16, 32) was the first step in exploring the use of microbial activity in biodegradation. That the activity is not confined to any one habitat is now apparent; the activity is found in several anaerobic environments (References 2, 18, 20, 25, 27, 33, 34). Although the rate of activity in sediment is low (Reference 2) compared to anaerobic sludge (References 2, 33), it seems as if the potential is greater than once believed (Reference 2).

Whereas the removal of the ortho chlorine from substituted phenols is certain (Figure 1), the possibility that this catalysis occurs by a nonenzymatic agent does exist (Reference 33). However, because dehalogenation is known to be biologically mediated (Reference 15) it seems this sediment activity is also. The herbicide 2,4,5-T appears to be dehalogenated in a manner similar to that seen in the 3-chlorobenzoate-degrading enrichment (Reference 35) yet different than in whole sludge, where cleavage of the ether linkage was predominant (Reference 19). With longer incubation periods and lower substrate concentration, the lake sediments will prove to be richer in dehalogenating activity than earlier believed.

The sediment enrichment is interesting for several reasons. First, the enrichment is more active at 37° than at 20°C (Table 4). Sediments themselves show little or no activity on 800 μM 3-F-, 3-Cl-, 3-B-, or 3,5-dichlorobenzoate at 7°C (<u>in situ</u> temperature) or at 55-67°C. Dehalogenation in temperate lakes may be limited or seasonal due to low temperature, making it difficult for some environments to detoxify themselves. The upper temperature limit is also interesting. Given that thermophilic methanogens (Reference 36), sulfidogens (Reference 37), and proton reducers (Reference 38) exist, dehalogenating organisms may also exist at elevated temperatures. However, under these experimental conditions (55-67°C) this was not the case. Several points should be considered in devising future thermophilic experiments: the choice of haloaromatic, the concentration of substrate and sediment, and the inoculum source. The opportunity for thermophilic dehalogenation should be better in whole sludges or in thermophilic environments than in temperate, dimictic lake sediments.

The sediment enrichment produced some new activities and specificities different from the sludge enrichment (Table 5). The conversion of 2-fluorobenzoate to CH_4 was quantitatively equal to that of 3-chlorobenzoate but the mechanism was not clarified. Defluorination may occur but benzoate was not identified by HPLC as a product. The fortuitous defluorination of 2-fluorobenzoate has been seen previously (References 39, 40). Given the small size of fluorine, the benzoate-degrading population could utilize the

fluorinated compound. In this case the molecule would either be defluorinated at a later metabolic step or a fluorinated product would form and accumulate. The overall sediment enrichment activity seems to be greater than that seen in the sludge enrichment. First, the possibility of dehalogenating more than one isomer of a given haloaromatic is an improvement when compared to activity that is confined to an individual isomer. Second, the expressed activity indicates that a different species is present in sediments than in sludge (References 19, 33).

The possibility that a dehalogenating species exists that is different from the 3-chlorobenzoate-degrading strain isolated from sludge (Reference 1) encouraged the extended isolation efforts. Using the roll tube technique described above, several isolates were obtained. However, none possessed the capacity to dehalogenate, and no organism was found to exhibit the distinct morphology of DCB-1. The difficulty in isolating this organism further argues for its individuality, as does the fact that conditions used to isolate DCB-1 failed to support the growth of the sediment organism. The dehalogenation reaction appears to be a fortuitous one, whereby the responsible organism(s) exist in low numbers or exist under the burden of obligate syntrophy. Isolation from this type of arrangement is not impossible but appears to demand special efforts. Due to the uniqueness of the organism and reaction, however, its isolation will be beneficial.

SECTION III

THE CHARACTERIZATION OF A UNIQUE SULFIDOGENIC ISOLATE THAT REDUCTIVELY DEHALOGENATES HALOAROMATIC COMPOUNDS

A. RATIONALE AND APPROACH

The dehalogenating isolate, strain DCB-1, previously isolated appeared to be a new and very unusual organism. Furthermore, its growth rate was slow and its yield poor. In order to obtain better growth rates and thus, improved dechlorination activity, it was necessary to better understand the physiology of this organism. If there were better carbon and energy sources, and/or if alternative electron acceptors could be used by DCB-1, the growth of this organism might be greatly improved. This section answers these and related questions on DCB-1 physiology.

B. MATERIALS AND METHODS

1. Organism and Culture Conditions

Strain DCB-1 (Reference 1) was used in these studies and grown on the basal salt medium described earlier (Reference 1). All pure culture work was performed under an 80 percent N_2 - 20 percent CO_2 atmosphere. Unless stated otherwise, clarified rumen fluid (centrifuged at 16300 X for 20 minutes) was added to the above salt medium. Where described, electron acceptors were added to achieve 2, 5, 10 or 20 mM concentration. P-5 medium consisted of the basal salt medium, 0.2 percent pyruvate and 5 mM fumarate. X-2 medium consisted of basal salt medium plus 0.2 percent pyruvate and various mM concentrations of thiosulfate. Yeast extract, when used for culture maintenance, was added to the basal medium at a concentration of 0.1 percent. The media were reduced either by 0.5 mM cysteine hydrochloride and 0.5 mM $Na_2S \cdot 9H_2O$ (cys-SH) or by 0.5 mM $Na_2S \cdot 9H_2O$ (SH) alone.

Dehalogenation tests were performed in 20 percent rumen fluid and 0.2 percent pyruvate (20.2 medium), whereas general maintenance of the organism was in 10 percent rumen fluid and 0.2 percent pyruvate (10.2 medium). Incubations were static, in the dark and at 37°C except when specified otherwise. Ten percent inoculations were made from 14-day-old cultures grown in 10.2 medium. For experiments where resuspended cells were used, the technique for establishing an inoculum was the same. A volume of cells (200-400 mL) was centrifuged at 16300 x gram anaerobically under N_2, the pellet and centrifuge flasks rinsed in fresh medium, and the pellet resuspended in 60-120 mL of that identical medium. Ten percent inoculations were made from this resuspended culture.

2. Growth Studies

Growth was monitored by measuring the optical density in 18 mm diameter anaerobic culture tubes using a Turner Model 350 spectrophotometer set at 66 nm. Growth on the various substrates was measured in the presence

or absence of 10 percent clarified rumen fluid. Carbon sources were used at concentrations of 0.1 percent to 0.3 percent, whereas growth factor additions ranged from 0.01 percent to 0.2 percent. These growth substrates were added before autoclaving or filter-sterilized by addition to tubes previously sterilized. If necessary, pH adjustments were made before autoclaving by the addition of dilute NaOH or HCl. After media sterilization gas additions were made to individual tubes by filtration through syringe amounted 0.45 μM Millipore filters. Transfers were made from 14-day-old cultures.

Coefficients of variation were determined for the 87 data points Figures 3, 4, 5, 6, and 7. Values ranged for 1.0 to 36.0 with a mean of 10.1 ± 6.9. Two criteria were used to define significant growth: (1) growth above background and (2) maintenance of growth on a particular substrate by repeated transfers to medium containing the same substrate. Where growth exceeded a certain set value, maintenance was tried. For fermentative growth in the absence of an electron acceptor, a growth curve below 0.02 OD units was considered negative. For growth on rumen fluid plus thiosulfate, growth had to exceed that obtained on rumen fluid minus any carbon source. Finally, for growth on substrates plus thiosulfate but lacking rumen fluid, a change in optical density of 0.05 or less was considered negative.

3. Equipment

Hydrogen concentrations were monitored on a Carle Analytical Gas Chromatograph Model 111 equipped with a 3 mL gas sampling loop. Carrier gas was pure argon-delivered at 20 mL/min through a combination of two columns: a 1.22 meter Porapak® T 50/80 mesh attached to a 2.74 meter Molecular Sieve 5A 45/60 mesh. HPLC analysis was performed on a Varian Model 5000 HPLC coupled to a Hitachi Model 100-40 spectrophotometer with an attached Altex® 100-55 spectrophotometer flow cell. Dehalogenation was monitored on a Hibar LiChrosorb, 10 μm particle size, RP-18 column. The wavelength varied for each compound tested.

The organic acids from intermediary metabolism were analyzed by HPLC on a Biorad Aminiex Ion Exclusion HPX-87H organic acid analysis column. Mobile phases used on the Hibar column were variations of either 50 mM acetate pH 4.5 diluted by acetonitrile or methanol, or a 70:40:13 (H_2O:CH_3OH:CH_3COOH) mixture diluted with methanol. For the HPX-87H column 0.008N H_2SO_4 was used with the detector set at 210 nm. All experiments for HPLC analysis were performed in triplicate.

4. Chemical Analyses

Protein concentrations were assayed by the method of Lowry et al. (Reference 41). Polysaccharides and glycogen were determined by the methods outlines in the Manual of Methods for General Bacteriology (Reference 42). Cytochromes were determined as previously described (Reference 43). For these analyses, test cells were centrifuged anaerobically, resuspended in phosphate buffer pH 7.0, ruptured in a French pressure cell at 9 x 10^7 Pa, and recentrifuged at 16300 x gram for 20 minutes. The supernatant was analyzed in a Perkin Elmer Model 350 double beam spectrophotometer scanning from 730 to 180 nm on a scale of .0.01 to 0.09.

Hydrogen sulfide was quantified by the Pachmayr method as described by Brock (Reference 44). Sulfite reductase (desulfoviridin) was qualified by a spectrophotometer and by the Postgate procedure (Reference 45). Desulfovibrio strains PS-1 and DG-II (gift from D. Dwyer) served as positive controls for the cytochrome analysis and sulfite reductase tests whereas Methanospirillum strain PM-1 (Reference 1), and the butyrate oxidizer strain NSF-1 (Reference 1) served as negative controls for the Postgate test.

C. RESULTS AND DISCUSSION

Because of the poor growth of DCB-1 [maximum growth of 0.05 O.D. (Reference 1)], the effects of various concentrations of rumen fluid and pyruvate as well as trypticase and pyruvate, were tested for rate of growth and final cell yield. No significant difference was detected between the combinations tried. After 17 days, the change in optical density readings for 10 percent and 20 percent rumen fluid plus 0.05, 0.1 and 0.2 percent pyruvate were all 0.02. No combination of trypticase and pyruvate supported growth resulting in optical density (O.D.) readings greater than 0.015. As a result 10.2 medium (10 percent R.F. + 0.2 percent pyruvate) was used for growth studies. The choice of 10 percent rumen fluid over 20 percent rumen fluid resulted in less background color for optical density readings.

Electron acceptors were also examined as a route to improve growth. When sulfur-based electron acceptors were added at 20 mM to 10.2 medium, thiosulfate greatly stimulated growth (Figure 3). In view of the possible toxicity of some of these anions, lower concentrations were also tested (Figure 4). The enhanced growth in tubes containing sulfite and thiosulfate was obvious, whereas the slow growth in the presence of the sulfate was surprising. Thiosulfate was the least toxic of the electron acceptors tested. Growth could not be maintained in X-2 media when nitrate or sulfate were substituted for thiosulfate as the electron acceptor, but growth was maintained in identical medium with sulfite. Growth in P-5 medium was poor.

Over 50 were surveyed to determine whether they supported growth by fermentation or with thiosulfate as the electron acceptor (Table 6). Based on the criteria outlined above, few of the 53 substrates showed any significant growth increases, and growth could only be maintained on pyruvate as a carbon source. No substrates, except pyruvate, were tested with any other electron acceptor.

Fermentation results (Figure 5) confirmed the previous work (Reference 1) that indicated the substrate range was limited. However, some growth occurred with thiosulfate in 10.2 medium with lactate and D-arabinose (Figure 6). Low amounts of growth, such as shown for glucose (Figure 5), were often difficult to distinguish as to whether they were supporting significant growth. In the medium containing thiosulfate, growth in the presence of many carbon sources appeared to occur at minimal levels.

Centrifuged and resuspended cells were used as inocula to determine the influence of rumen fluid on growth (Figure 7). Whereas growth with pyruvate was normal, as was the lack of significant growth in rumen fluid alone, the

TABLE 6. SUBSTRATES TESTED WHICH WERE INCAPABLE OF SUPPORTING GROWTH WITH OR WITHOUT THIOSULFATE[a].

H_2:CO_2	c	NaCitrate		ETOH	c
Acetate:H_2	c	D(-)ribose	b	Benzoate	
Acetate:N_2	c	-L-rhamnose		Phenol	c
L-arabinose		D(+)-trehalose		Anisole	c
D-arabinose		Ribitol	b	Succinate	c
L-malate		Pectin	b		
D-malate		Malonic Acid	b		
formate		Fumarate	a		
choline		D-gluconic acid	b		
Na Lactate		D-glucuronic acid	b		
glycerol		amylose	b		
D(+)mannose		cellobiose			
maltose		lactose			
sucrose		oxalate			
D-glucose		i-inositol	b		
D + galactose		inulin	b		
-D(-)fructose		soluble starch			
D-Manitol	b	valeric acid	c		
DL-arabitol		caproic acid	c		
-alanine		iso.valeric acid	c		
N-acetylglycine	b	crotonic acid	c		
L-ascorbic	b	butyric acid	c		
D(+) Sorbitol	b	isobutyric acid	c		
D(+) Xylose		propionic acid	c		

[a]Tested with and without thiosulfate except, a = tested only with thiosulfate and b = tested only without thiosulfate.

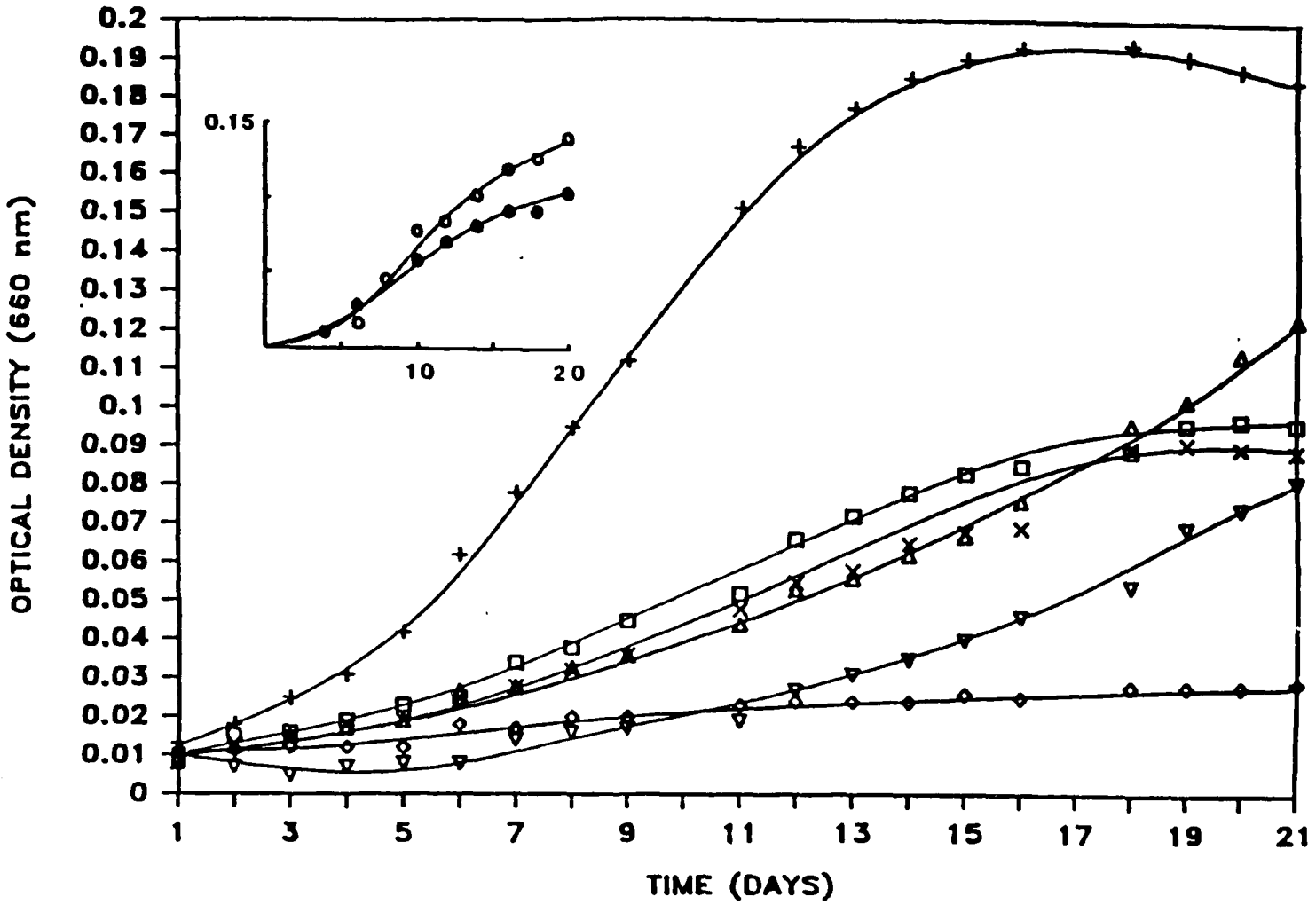

Figure 3. Growth of DCB-1 with various electron acceptors in 20.2 medium. SYMBOLS: crosses, 20 mM $S_2O_3^=$; squares, no electron acceptors; triangles, 20 mM $SO_4^=$; X's 10 mM NO_3^-; inverted triangles, S^o; diamonds, 20 mM $SO_3^=$. Each data point represents the mean of two experiments comprised of five test tubes each. INSET: Growth of DCB-1 with fumarate, SYMBOLS: open circles, no electron acceptor; closed circles, 5 mM fumarate. Each data point is the mean of values from five test tubes.

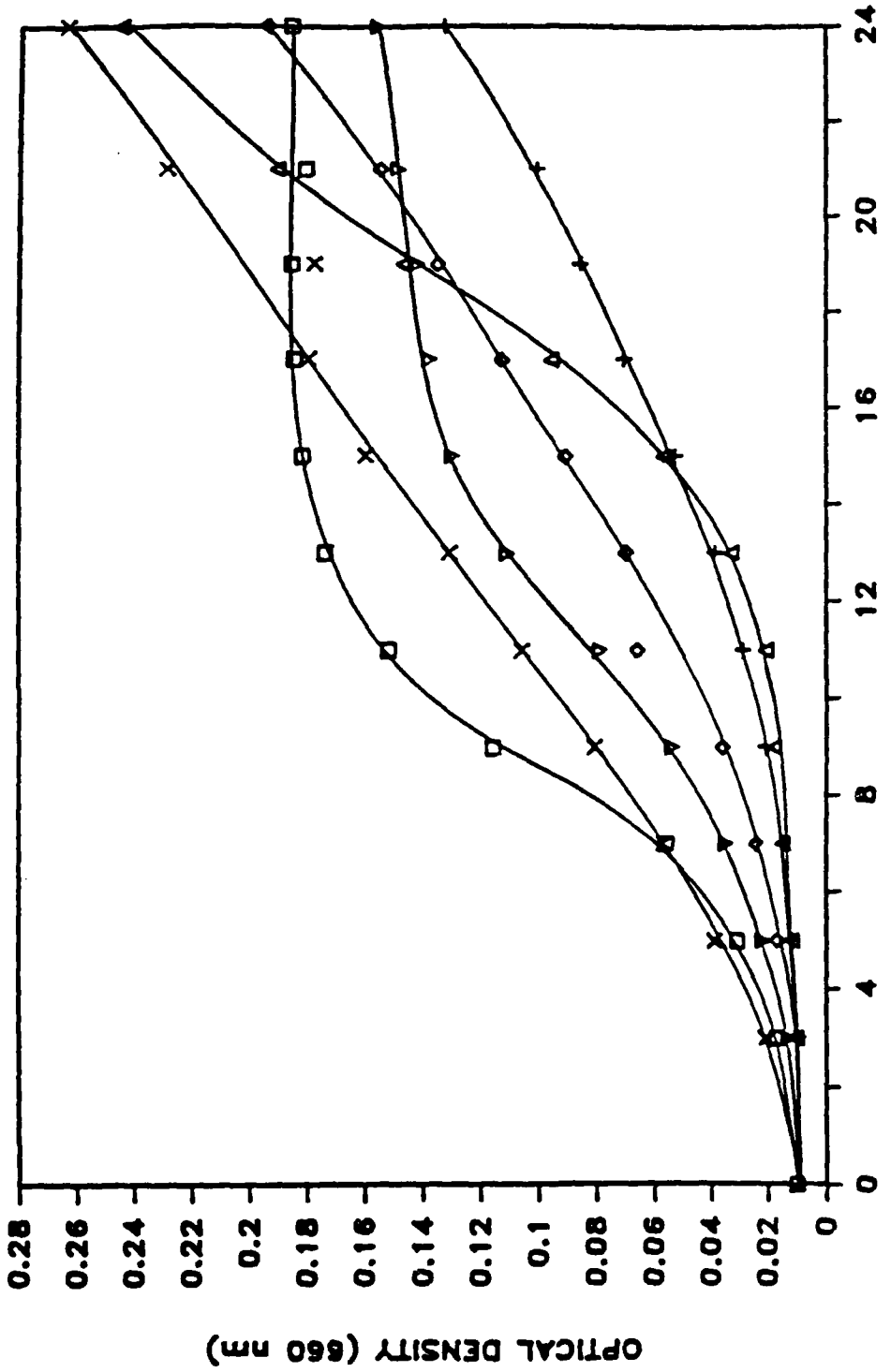

Figure 4. Effect of various oxygenated sulfur electron acceptors on the growth of DCB-1 in 20.2 medium. SYMBOLS: squares, 20 mM $S_2O_3^=$; X's, 5 mM $SO_3^=$; triangles, 10 mM $SO_3^=$; diamonds, 2 mM $SO_4^=$; crosses, 10 mM $SO_4^=$; inverted triangles, no electron acceptor. Each data point represents the mean of two experiments comprised of five test tubes each.

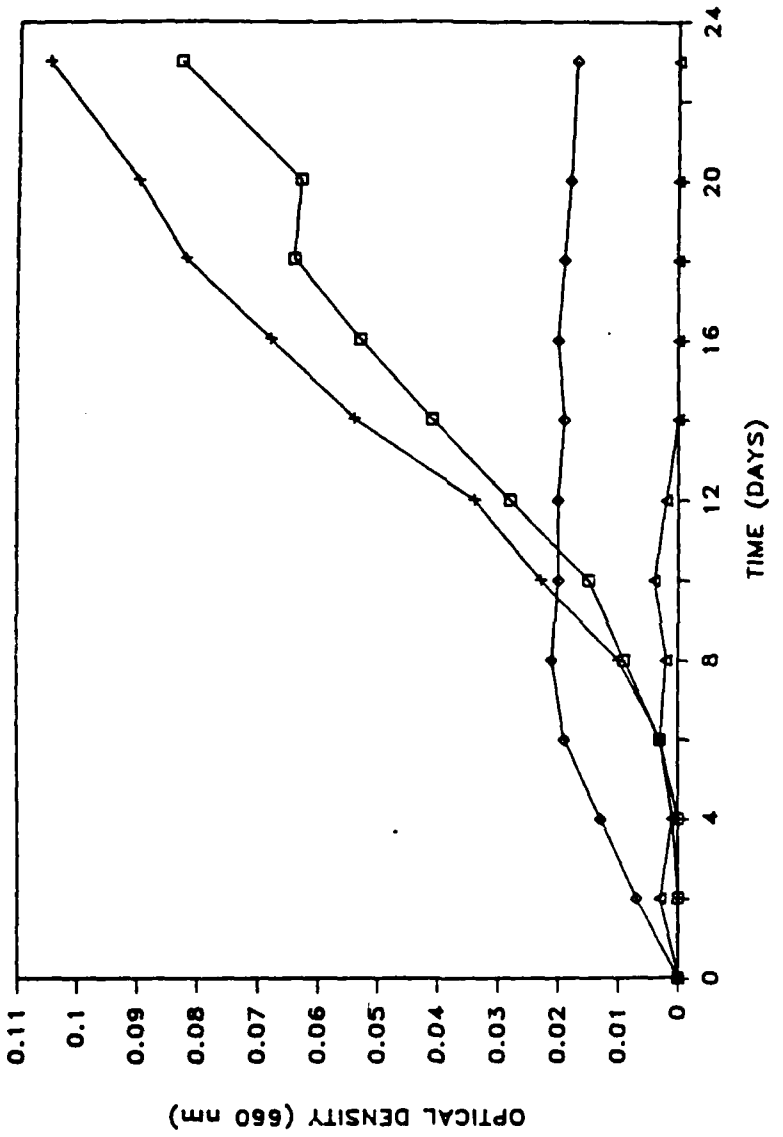

Figure 5. Growth of DCB-1 on 10 Percent Rumen Fluid and Various Carbon Sources in the Absence of Thiosulfate. SYMBOLS: Crosses, Autoclaved Pyruvate; Squares, Filtered Pyruvate; Diamonds, Glucose; Triangles, L-arabinose. Each Data Point is the Mean of Values from Four Test Tubes.

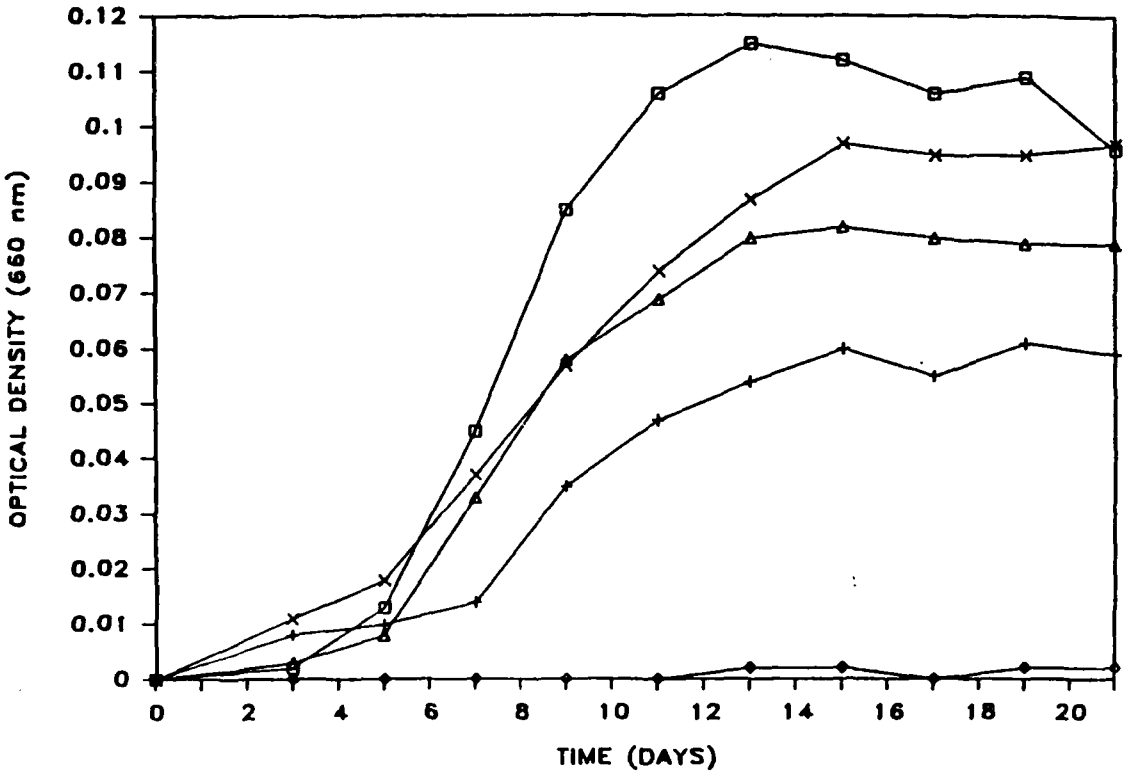

Figure 6. Growth of DCB-1 on 10 Percent Rumen Fluid, 20 mM $S_2O_3^=$ and Various Carbon Sources. SYBMOLS: Squares, Pyruvate; X's, D-arabinose; Triangles, Acetate; Crosses, Lactate; Diamonds, H_2:CO_2. Each Data Point is the Mean of Values from Four Test Tubes.

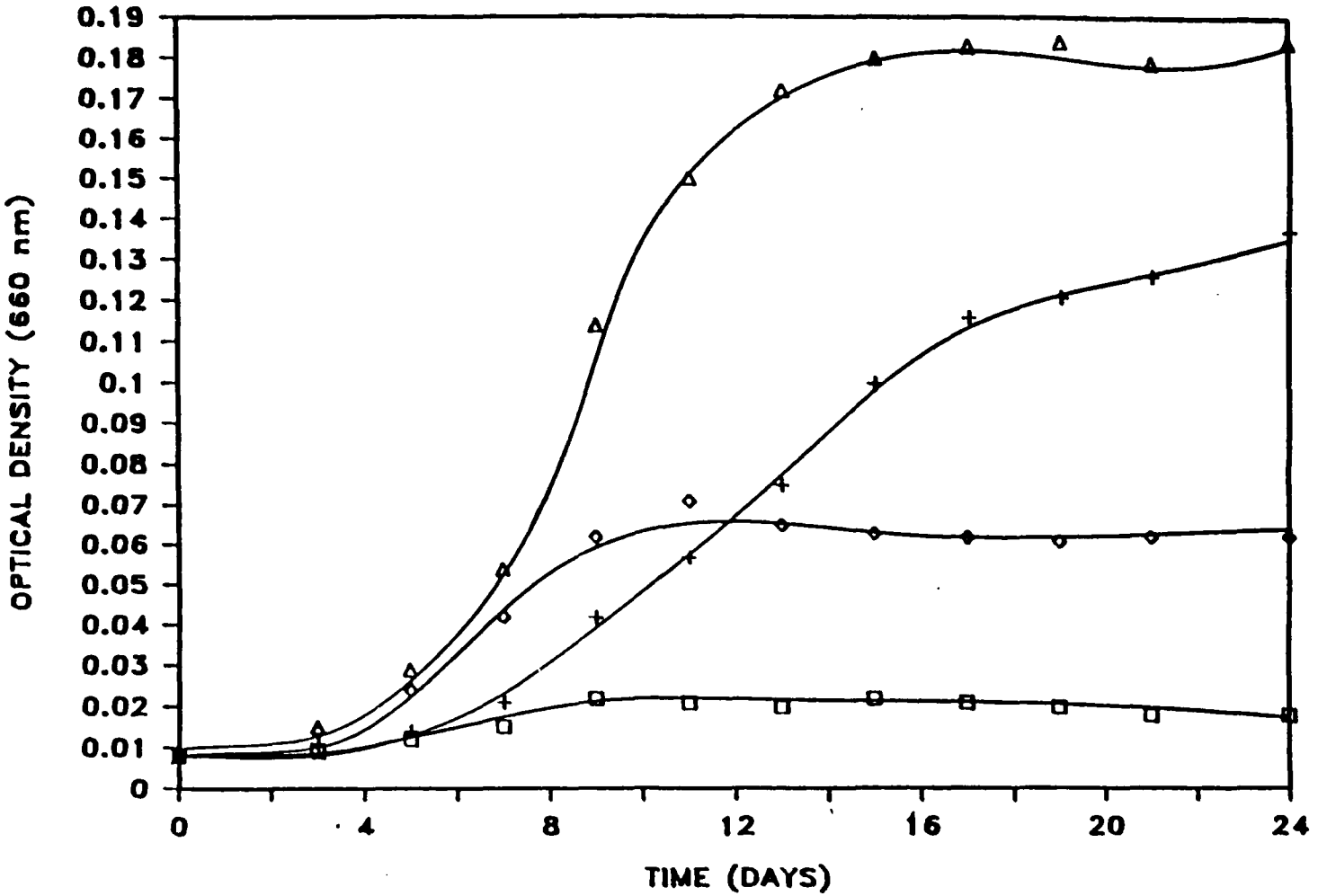

Figure 7. Growth of DCB-1 with 10% rumen fluid, pyruvate and $S_2O_3^=$. INOCULUM: centrifuged and washed cells resuspended in 10% rumen fluid. SYMBOLS: triangles, 0.2% pyruvate + 20 mM $S_2O_3^=$ + 10% rumen fluid; crosses, 0.2% pyruvate + 10% rumen fluid; diamonds, 20 mM $S_2O_3^=$ + 10% rumen fluid; squares, 10% rumen fluid only. Each data point is the mean of values from five test tubes .

growth with rumen fluid and thiosulfate helped explain the results with lactate and D-arabinose. Tests in basal salt medium containing thiosulfate, carbon sources and no rumen fluid confirmed that only pyruvate supported significant growth, with or without an electron acceptor (Figure 8). Poor growth on acetate plus hydrogen and thiosulfate was maintained through several transfers. Growth on glycerol, L&D arabinose, and L&D malate was greater than growth on rumen fluid alone. However, this growth could not be maintained with repeated transfer.

Because cysteine was used as a medium reductant, we determined if cysteine influenced growth. Washed and resuspended cells were used to inoculate the media in the presence of cysteine-sulfide (0.52 mM) as reductants. Because growth was not significantly different in the presence or absence of cysteine, it was concluded that cysteine was not used as a carbon source.

Isolate DCB-1 consumed hydrogen, both during pyruvate fermentation and thiosulfate reduction, but the rate of consumption was twice as fast on growth during thiosulfate reduction. In 20.2 medium, the presence of SH in place of cys-SH or SH + 3-Cl-BZ had little influence on hydrogen consumption. The presence of H_2 in the headspace interfered with growth. In 21 days the optical density of the culture grown under N_2:CO_2 was 0.03 units greater than those grown under H_2:CO_2. Analysis of the headspace gas over several days did not reveal any hydrogen production (hydrogen detection limit was 10 ppm) during the early stages of growth on 20.2 medium.

The cytochrome spectrum of DCB-1 matched that of Desulfovibrio strain DG-II (Figure 9) and was characteristic of cytochrome c with absorption maxima at 420, 521, and 553 nm. A peak was identified as sulfite reductase (630 nm) which was confirmed by an identical peak in strain DG-II (Figure 10). Both Desulfovibrio strains PS-1 and DCB-1 tested positive for desulfoviridin by the Postgate test whereas two other obligate anaerobes. Methanospirillum PM-1 and an anaerobic butyrate oxidizer, strain NSF-1, tested negative.

Polysaccharides and glycogen were easily extracted with glycogen presumably coming from the dark bodies seen in transmission electron microscopy pictures (Reference 1). Glycogen represented approximately 80 percent of the extractable polysaccharide.

Using centrifuged and resuspended cells for inoculation. the growth on acetate in the presence of thiosulfate was measured with the concurrent utilization of acetate. In 28 days under a nitrogen atmosphere, acetate levels dropped from 12.4 mM to 12.2 mM with the production of 239 µg/mL of protein. During this period 0.9 mM of SH- were formed. During the same time span under a hydrogen atmosphere, acetate levels increased from 12.5 mM to 23.2 mM with the production of 347 µg/mL of protein. During this period 0.7 mM of SH- were formed. These cultures also were characterized by the presence of 4.8 mM of formate and 1.1 mM succinate.

A fermentation balance was attempted for DCB-1 growing on pyruvate in the presence of thiosulfate and fumarate (Table 7). In the presence of fumarate, the rate of pyruvate consumption was slightly higher than in thiosulfate

TABLE 7. CARBON COMPOUNDS IDENTIFIED DURING GROWTH ON DCB-1 UNDER DIFFERENT CULTURE CONDITIONS.

Medium and Time	Pyruvate	Acetate	SH-	Fumarate	Lactate	Succinate	Protein
(days)	mM						(µg/mL)
20.2 + mM S_2O_3 = + N_2:CO_2							
T = 0	10.0	<1	-	0	0	-	1065
T = 28	4.5	7.5	0.8	0	0.4	-	1181
5 mM Fumarate + 80 percent N_2:20 percent CO_2							
T = 0	8.3	0	0	5.4	-	0	305
T = 28	1.3	6.8	0	4.6	-	0.8	191
5 mM Fumarate + 80 percent H_2:20 percent CO_2							
T = 0	6.6	0	0	5.0	-	0	262
T = 28	0.6	5.9	7.1	4.8	-	3.1	163

[a]Results of triplicate tests.

medium yet there was only an apparent reduction of 800 micromoles of fumarate to succinate under an N_2:CO_2 atmosphere. Under an H_2:CO_2 atmosphere in media containing fumarate, formate and additional succinate were formed by extraneous reactions, yet in both an N_2:CO_2 atmosphere and an H_2:CO_2 atmosphere the conversion of pyruvate to acetate was stoichiometric. The consumption of pyruvate to acetate in the presence of 2.5 mM thiosulfate was 109 percent but other products were also formed at 1 mM levels or greater (Figure 11). The production of HPLC peaks representing unknown carbon compounds was also seen during carbon studies with acetate under N_2:CO_2 and H_2:CO_2 atmosphere (data not shown). The presence of these peaks indicate that growth involves more than the simple conversion of pyruvate to acetate.

Hydrogen sulfide yields from 1 mM and 2 mM thiosulfate containing cultures were determined after 14 days. The sulfide yields were 0.48 and 0.36 mM, respectively, indicating the slow usage of the electron acceptor. Using known sulfide standards (2 mM, 3 mM and 4 mM samples) in phosphate buffer, pH 7.0, sulfide recoveries of 96 percent, 99 percent, and 90 percent, respectively, were obtained.

On the assumption that the organism was a unique sulfidogen, four strains of Desulfovibrio: PS-1 (Reference 1), DG-II (gift from D. Dwyer), and DDS and G11 (gifts from H. Peck) were tested for their ability to dehalogenate 3-Cl- and 3-bromobenzoate under fermentative conditions. Whereas growth was excellent, none of the strains indicated a dehalogenation capacity in 20.2 medium.

No carbon source except pyruvate supported growth. Identifying thiosulfate as a potential electron acceptor increased the opportunity that other utilizable substrates existed because sulfidogenic bacteria have a specialized but diverse range of substrates that support growth (References 46, 47, 48, 49, 50, 51, 52). Because thermodynamic barriers are often removed when oxygenated sulfur electron acceptors are employed as electron sinks (Reference 53), even compounds such as acetate (References 48, 49) and fatty acids (References 47, 49, 51) are utilized for growth. DCB-1 used only pyruvate, with or without an electron acceptor, in chemically defined media.

In the presence of rumen fluid, most carbon sources appeared to support growth as did rumen fluid alone. The assumption was that acetate supported growth, yet growth with acetate in a chemically defined medium was poor. Therefore, either acetate was not supporting growth or, in addition to acetate as a carbon source, other growth factors were present which were removed from the defined medium by the removal of rumen fluid. Routine transfers now contain H_2:CO_2 atmosphere, but growth continues to decrease.

Due to the lack of growth in defined media (Figure 8), it was assumed that earlier growth (Figure 6) was not real. However, if growth factors in rumen fluid are needed, then growth for some carbon sources might have been real. In particular arabinose and malate supported a level of growth that was slightly better than that maintained by rumen fluid alone. Whereas arabinose is an unlikely substrate for a sulfate reducer given the substrate range of

other sulfate reducing species (Reference 52), malate would be logical because many sulfidogenic organisms are capable of its utilization.

Sulfate appeared to be a poor electron sink, yet sulfite and thiosulfate supported growth on pyruvate in basal salt medium, and sulfite reductase, desulfoviridin, was present as determined by comparison to the known Desulfovibrio sp., DG-II. Sulfate would not support growth in defined basal salt medium, would not support rapid growth significantly in excess of pyruvate fermentation in rumen fluid, and produced little sulfite.

Three explanations are possible. First, the organism may be incapable of expending the adenosine triphosphate (ATP) necessary to form adenophosphosulfate (APS) or lack APS sulfurylase (Reference 54). Neither sulfite nor thiosulfate use would be influenced by this metabolic deficiency. Second, the enzymes necessary for sulfite and thiosulfate reduction may be constitutive whereas the ATP sulfurylase is inducible. This would explain the long lag period preceding growth with sulfate in rumen fluid but not the lack of growth in defined medium. Finally, the organism could require a growth factor as an energy source for growth on sulfate only or maybe deficiency in APS reductase. That the organism is deficient in ATP sulfurylase or needs an energy source in conjunction with the induction of the requisite enzymes both seem plausible. Further work with labelled sulfate and sulfate reductase enzyme assays should help to clarify this problem.

Other electron acceptors were also tested. Whereas nitrate is reduced to nitrite (Reference 1), it would not support growth on pyruvate in basal salt medium or in Tryptic Soy Nitrate broth. Other sulfate reducing organisms reduce nitrate either in the presence of sulfate (Reference 55) or in its absence (Reference 56). Fumarate supported poor growth in the basal salt medium (P-5), but the addition of 0.01 percent yeast extract was ultimately needed to maintain the culture.

The basic problem then becomes defining what the ecological niche is. Eliminating pyruvate (and the other tested compounds as natural substrates. the situation becomes complex. The organism may exist as an integral member of a syntrophic relationship in sewage or, possibly, the natural substrate is a complex compound or substituted aromatic. In the latter case, the carbon metabolism may help explain the unique dehalogenating activity. Because dehalogenation is apparently fortuitous, resulting in the stoichiometric accumulation of the corresponding dehalogenated aromatic, the enzyme utilized in carbon metabolism may be responsible for the dehalogenation event.

Carbon studies indicate that this organism's metabolism is more complex than first believed. Whereas hydrogen is consumed by sulfidogenic organisms (Reference 46) which are used in coculture with proton reducing species (Reference 57), its influence on carbon assimilation was difficult to understand.

In the presence of thiosulfate, the formation of formic acid, succinic acid and several other intermediates by Day 28 suggests that bicarbonate is used during growth or that pyruvate acts to accept electrons. Whereas

fumarate is stoichiometrically converted to succinate (800 μM), growth is poor and several intermediate carbon forms develop in the presence of hydrogen.

Protein data for the pyruvate thiosulfate experiments indicate that growth occurred corresponding to the conversion of pyruvate to acetate. This is confirmed by the production of 800 micromoles of sulfide. The protein data for the fumarate-pyruvate tests is more difficult to interpret. Death and protein denaturation may be the cause of the loss of protein seen after 28 days. Earlier sampling may show protein increases. A second cause may be that the reduction of fumarate to succinate is not coupled to growth.

The nonstoichiometric conversion of pyruvate to acetate with the corresponding reduction of thiosulfate to hydrogen sulfide leaves a hydrogen and carbon imbalance. The 2.5 mM thiosulfate and 10 mM pyruvate are incompletely utilized. If the hydrogenases are a typical enzymes, as results indicate by the lack of detectable hydrogen production, and the sulfide production does not account for the difference in hydrogen, then the fate of the hydrogen must be explained.

Reactions involving hydrogen, bicarbonate, acetate, and pyruvate are thermodynamically favorable and may help to explain the metabolism as well as the aberrant peaks that develop upon incubation (Figure 9). Whereas these reactions or others (Reference 53) may be occurring, several of the peaks remain unidentified. Most notable are the peaks developing at 17:70 and 23:62 which do not correspond to proportionate or butyrate standards yet should be similar size molecules.

Many facets of this organism's metabolism remain enigmatic, and several questions remain as to the role this organism occupies in nature. Whereas several aspects of its growth and existence remain unexplained, its uniqueness justifies further work. Exploitation of its metabolic pathways, dechlorinating ability, hydrogen consuming capacity, and genetics will result in new scientific prospects.

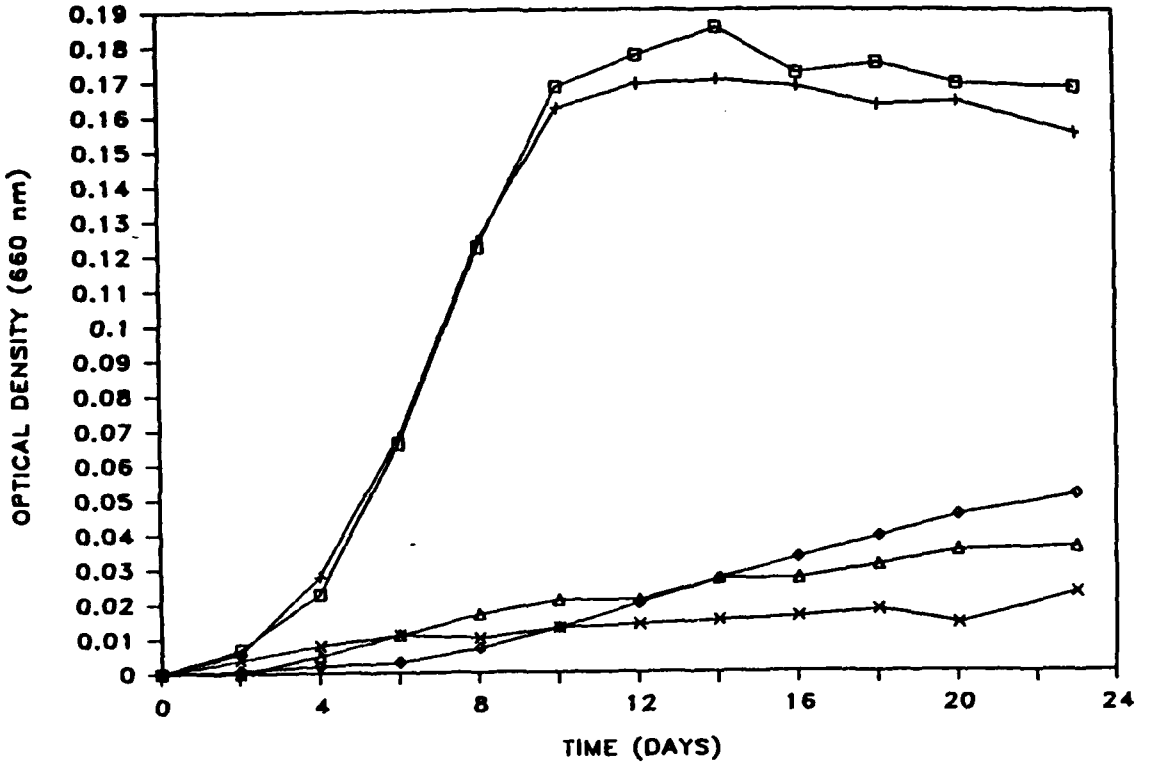

Figure 8. Growth of DCB-1 on Mineral Salts, 20 mM $S_2O_3^-$ and Various Carbon Sources. SYMBOLS: Squares, Filtered Pyruvate, Crosses, Autoclaved Pyruvate; Diamonds, Acetate; Triangles, H_2:CO_2 x's D-arabinose. Each Data Point is the Mean of Values from Four Test Tubes.

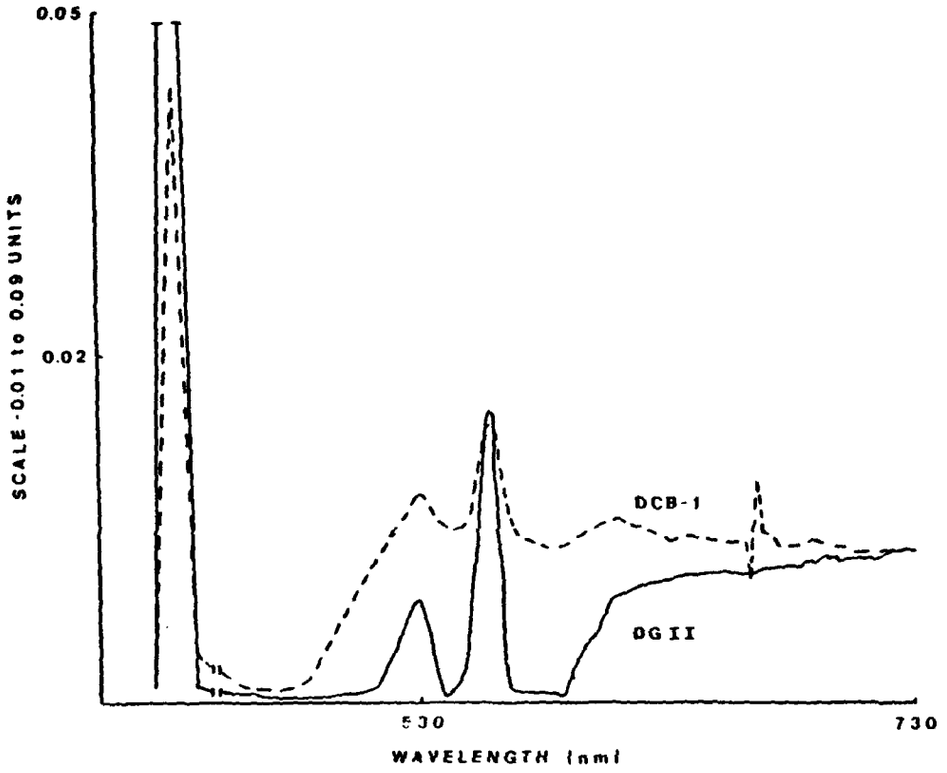

Figure 9. Evidence of cytochrome c in DCB-1 Grown on Thiosulfate, Pyruvate and Mineral Salts. Solid Line: DG II Control; Dotted Line, DCB-1.

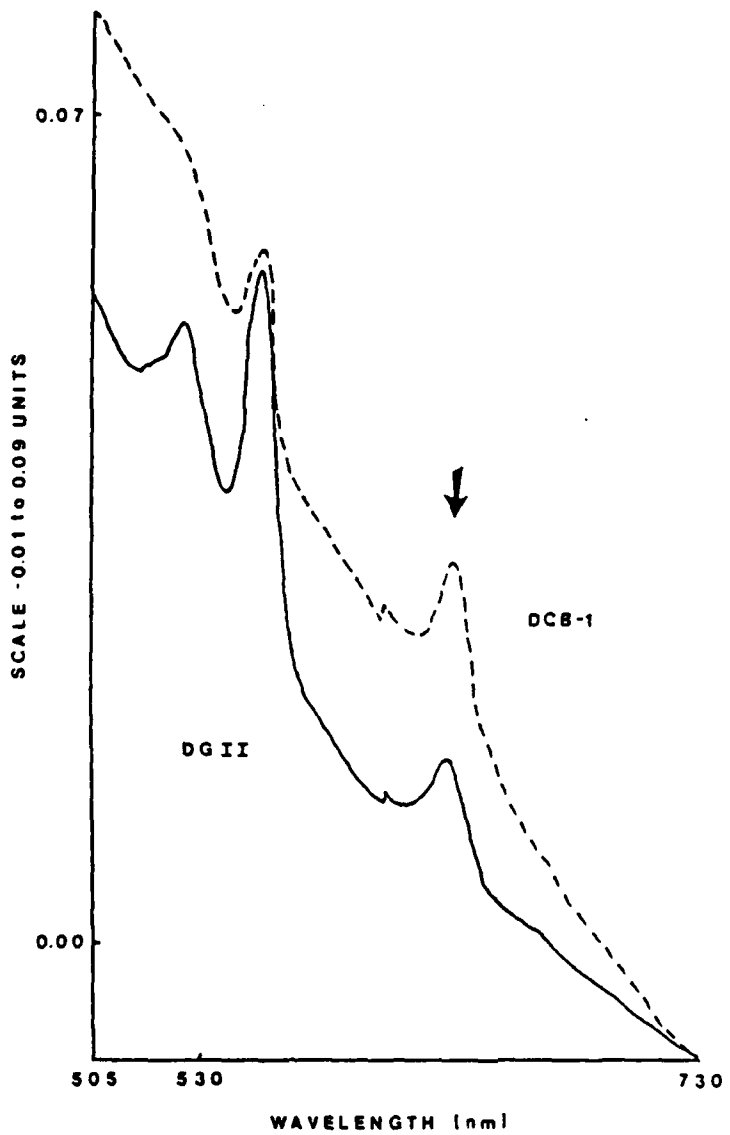

Figure 10. Evidence of Sulfite Reductase (Desulfoviridin) in DCB-1 Grown on Thiosulfate, Pyruvate and Mineral Salts. Solid Line, DG II Control; Dotted Line, DCB-1.

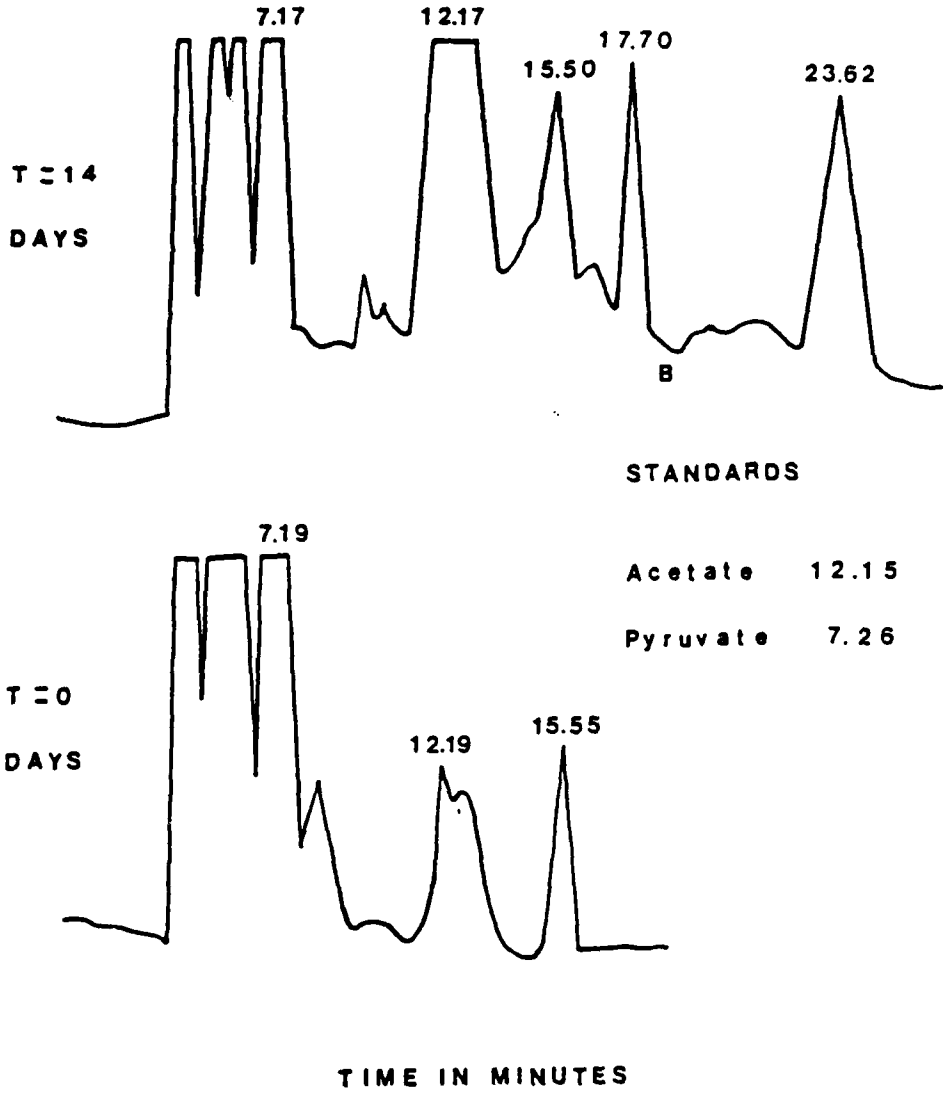

Figure 11. Carbon-containing products that develop in 14 days in cultures of DCB-1 when grown on pyruvate and thiosulfate.

SECTION IV

CHARACTERIZATON OF THE INFLUENCES ON DEHALOGENATION BY A SULFIDOGENIC DECHLORINATING BACTERIUM STRAIN DCB-1

A. RATIONALE AND APPROACH

Findings of the previous section showed that DCB-1 reduced oxidized sulfur compounds, consumed H_2, and benefited from the growth factors in rumen fluid. This section reports on whether the use of these compounds affects dechlorination. Furthermore, the range of halogenated benzoate substrates that metabolized was examined now that improved growth conditions had been established. This experiment also provided information on the rate of dehalogenation which is useful both to interpreting mechanisms and evaluating practical treatment schemes.

B. MATERIALS AND METHODS

1. Media and Culture

Strain DCB-1 was grown on basal salt medium but modified by the addition of clarified rumen fluid, trypticase or other nutrients as indcated. Pyruvate at 0.2 percent served as the carbon source, and the standard medium was basal salts, 20 percent rumen fluid and 0.2 percent pyruvate (20.2 medium). Electron acceptors were added at concentrations ranging from 5 to 20 mM. The gas phase was 80 percent N_2-20 percent CO_2. The media were reduced either by 0.5 mM cysteine hydrochloride and 0.5 mM $Na_2S \cdot 9H_20$ (cysteine-SH) or by 0.5 mM $Na_2S \cdot 9H_20$ alone. In experiments where pyruvate or ammonium concentrations were lowered, pyruvate was used at 0 02 percent and ammonium concentrations were lowered from 0.535 to 0.054 g/1. In these experiments the Ca, Mg, and Fe salts were evaluated at the two concentrations given (g/1): $CaCl_2$ $2H_20$, 0.015 and 0.735; $MgCl_2$:$6H_20$, 0.020 and 0.102; and $FeCl_2 \cdot 4H_20$, 0.004 and 0 02.

Halogenated benzoates and phenoxyacetates were prepared as Na^+ salts (Reference 58) whereas phenols were prepared in water (Reference 33). Quantities of these stock solutions were added to individual serum bottles after filtration through syringe-mounted 0.22 μm Millipore filter units to yield the desired concentrations. Chlorinated anilines and benzonitrile were dissolved in ethanol and added to the serum bottles without sterilization. Chloramphenicol (a final concentration of 20 μg/mL) was also added after filter sterilization.

Cultures were grown in 160 mL serum bottles at 37°C in the dark except where noted. Inoculation was by 10 percent transfers for 14-day-old cultures grown on the same medium used in the experiment. Unless otherwise noted each treatment was performed in triplicate. For zero time samples (353 μM ± 38 to 375 μM ± 18), the mean coefficient of variation was 6 percent ± 3 percent for five experiments comprised of triplicate bottles. For samples in the process of dehalogenation (173 μM ± 30 to 226 μM ± 12) the mean coefficient of variation was 14 percent ± 5 percent. Roll tubes were prepared as described in Chapter 3.

For experiments where resuspended cells were used, the technique for establishing an inoculum was the same. A volume of cells (200-400 mL) was centrifuged anaerobically under N_2, the pellet and centrifuge flask rinsed in fresh medium, and the pellet resuspended in 60-120 mL of that identical medium. Ten percent inoculations were made from this resuspended culture.

2. Analysis

Samples were taken aseptically, filtered through Millipore 0 45 µM filters and frozen. Some samples were filtered through Pall® filters, type Nylon-66, 0.45 m which were then washed with acetonitrile. Dehalogenation was monitored by a Varian® Model 5000 HPLC coupled to a Hitachi 100-40 spectrophotometer equipped with an Altex®155-00 spectrophotometer flow cell. Substrate and product peaks were identified by comparison to authentic standards. Data was collected either on a Hewlett Packard 3900A computing integrator or a Spectra Physics 4200 computing integrator. The column was Hibar LiChrosorb®, 10 m particle size, RP-18 and the mobile phase was either a variation of a 50 mM sodium acetate pH 4.5 system diluted with acetonitrile or methanol, or a 70:40:13 (H_2O:CH_3OH:CH_3COO) phase diluted with methanol. Flow rates varied to accomodate the particular compound. Products were identified by retention time using reagent grade compounds as the standard. Quantitation was done using standards prepared using the same compounds. Hydrogen levels were monitored on a Carle AGC-111 gas chromatograph equipped with a 3 mL gas sampling loop. Argon was the carrier gas set at a flow rate on 20 mL min^{-1}. Separation was by two columns: (1) a Porapak® T (1.2 m length, 1 3.2. mm i.d.), and (2) a Molecular Sieve 5A (2.7 m length, 3.2. mm i.d.). Detection was by a microthermistor. Protein concentrations were determined by the method described by Lowry et al. (Reference 41).

C. RESULTS AND DISCUSSION

1. Physical Influences on Biological Dehalogenation

Dehalogenation in the 3-chlorobenzoate enrichment (Reference 58) and in lake sediment was reported as biologically catalyzed. The isolation of strain DCB-1 (Reference 59) was further evidence of microbial mediation of haloaromatic dehalogenation. The pure culture was used to better define the biological basis and requirements of dechlorination. Anaerobiosis and 37°C were required for dehalogenation to occur (Figure 12); the same conditions that characterize the organism's natural (sludge) environment. Autoclaved cultures that were actively dehalogenating prior to sterilization and similarly treated cultures that had their gas phase changed from 80:20 N_2CO_2 to H_2:CO_2 after sterilization, all failed to initiate the reductive process (Figure 12). Most important hydrogen could not serve as an abiotic source of electrons.

To test if physical support -- such as might exist due to the presence of organic matter flocs in digester sludge -- would help dehalogenation, DCB-1 was grown in the presence of glass beads (Glasperlen, 1.00 to 1.05 mm diameter). However, no differences in dehalogenation rate or lag time were noted. As further evidence of biological activity, washed and concentrated

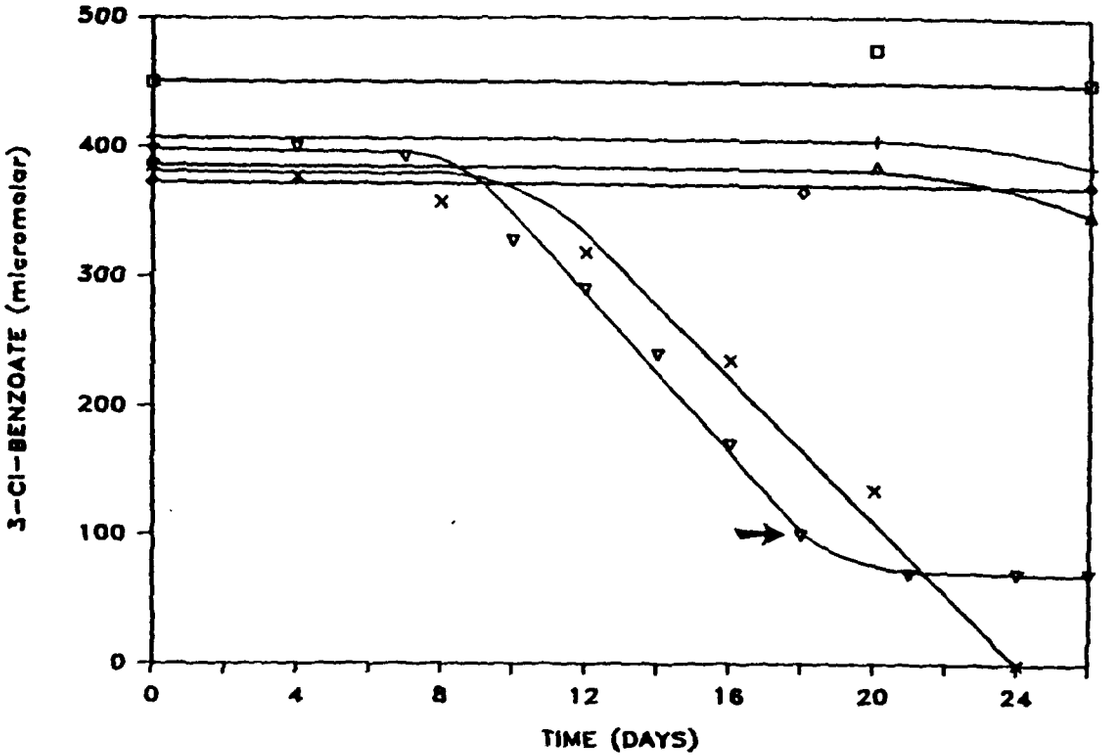

Figure 12. The Effects of Autoclaving, Temperature, and Air on Dehalogenation of 3-Chlorobenzoate by Strain DCB-1 in 20.2 Medium. SYMBOLS: Squares, 20 mM $S_2O_3^-$ Present at Day Zero; Crosses 20 mM $S_2O_3^-$ Added at Day 17 (Arrow); Diamonds, Cells Grown in 20 mM $S_2O_3^-$, Centrifuged, Washed, Resuspended, and Grown in the Absence of $S_2O_3^-$.

thiosulfate grown cells were used to inoculate fresh 20.2 media containing sulfanilamide (0.0025 percent) or chloramphenicol (20 μg/mL). No dehalogenation or growth was noted in either set of cultures with inhibitors, but it did occur in uninhibited cultures. The addition of chloramphenicol to actively dehalogenating cultures also stopped the reaction. The above results all support the concept that the process is only carried out by living cells under anaerobic conditions.

2. Effect of Thiosulfate and Other Electron Acceptors

Because growth of DCB-1 improved with the inclusion of thiosulfate in 20.2 medium, we examined whether the enhanced growth affected dehalogenation. Whereas thiosulfate increased growth rate, it was a strong inhibitor of dehalogenation whether present in the original medium or added to actively dehalogenating cultures that were grown by fermenting pyruvate (Figure 13). Thiosulfate concentrations of 5.0 mM or greater completely inhibited dechlorination (Figure 14). Cells grown in the presence of 20 mM thiosulfate and exhibiting no reductive dehalogenation during growth quickly regained the capacity to dehalogenate when washed free of thiosulfate and resuspended in new 20.2 medium (Figure 13).

To determine whether the inhibition by thiosulfate was a general effect of electron acceptors, other electron acceptors were used to assess if dehalogenation was also inhibited in their presence. Of the five electron acceptors studied, only the three sulfur compounds inhibited dehalogenation (Table 8). Thus, the presence of any electron acceptors was not sufficient to impede dehalogenation.

3. Effects of Organic and Inorganic Growth Factor on Dehalogenation

Carbon sources and growth factors were altered to determine if changes in any of these factors would enhance or inhibit dehalogenation. Rumen fluid in high concentrations of up to 20 percent (Table 8) was the most effective medium supplement aiding both growth and dechlorination (Table 8). Neither were further enhanced by trypticase or casamino acid additions. Trypticase could partially substitute for rumen fluid but concentrations above 0.01 percent began to inhibit dechlorination but not growth (Table 8). In the presence of rumen fluid 0.1 percent trypticase did not inhibit dechlorination. The batch of rumen fluid significantly altered the rate of dehalogenation and the time needed to dehalogenate a given quantity of 3-Cl-BZ (Figure 15). This made rate comparisons among different batches of rumen fluid more difficult. Nonetheless, rumen fluid at 20 percent was the most consistent medium for guaranteeing dehalogenation, and, thus, was used routinely in all media.

We also varied pyrvuate and ammonium by a factor of 10 below 20.2 medium to determine dehalogenation of 3-Cl-BZ was related to carbon or nitrogen nutrition. Little difference was seen in the time needed to dehalogenate 400 μm 3-Cl-BZ (19-23 days) or the rate of dehalogenation

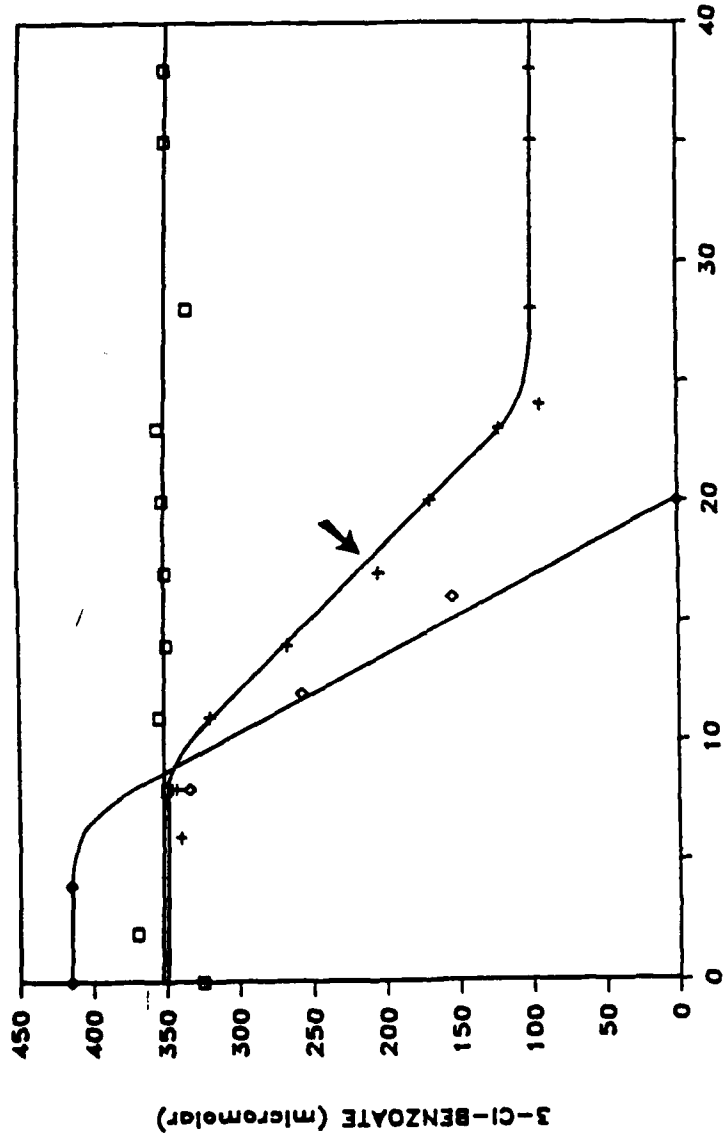

Figure 13. The Influence of Thiosulfate on Dehalogenation of 3-Chlorobenzoate by DCB-1 in 20.2 Medium.

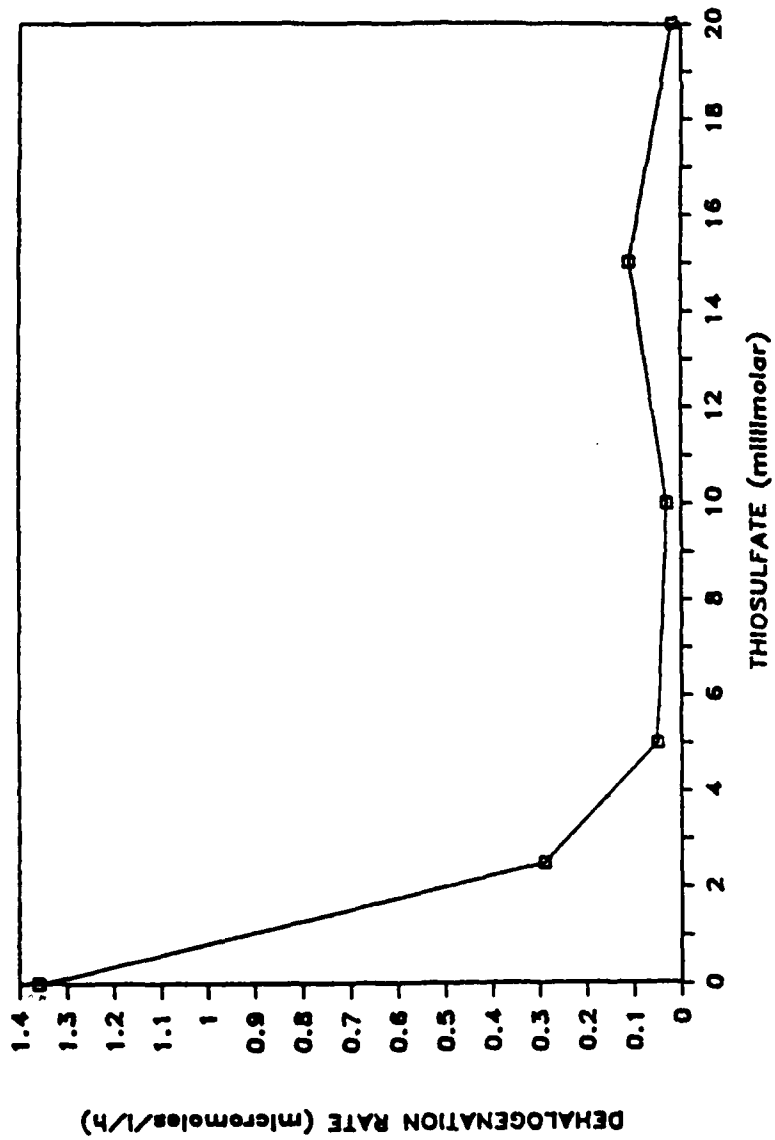

Figure 14. Effect of Thiosulfate Concentration on Rates of Dehalogenation of 3-Chlorobenzoate in 20.2 Medium.

TABLE 8. INFLUENCE OF SEVERAL ELECTRON ACCEPTORS ON DEHALOGENATION OF 3-Cl-BENZOATE IN 20 PERCENT RUMEN FLUID AND 0.2 PERCENT PYRUVATE.

Electron acceptor (5 mM)	Percent 3-Cl-benzoate remaining[a]	
	Day 20	Day 30
None	21	0
$S_2O_3^=$	71	64
$SO_3^=$	75	20
$SO_4^=$	57	17
NO_3^-	21	0
Fumarate	14	0

[a]Values are means of six bottles; two experiments of three 160 mL serum bottles each.

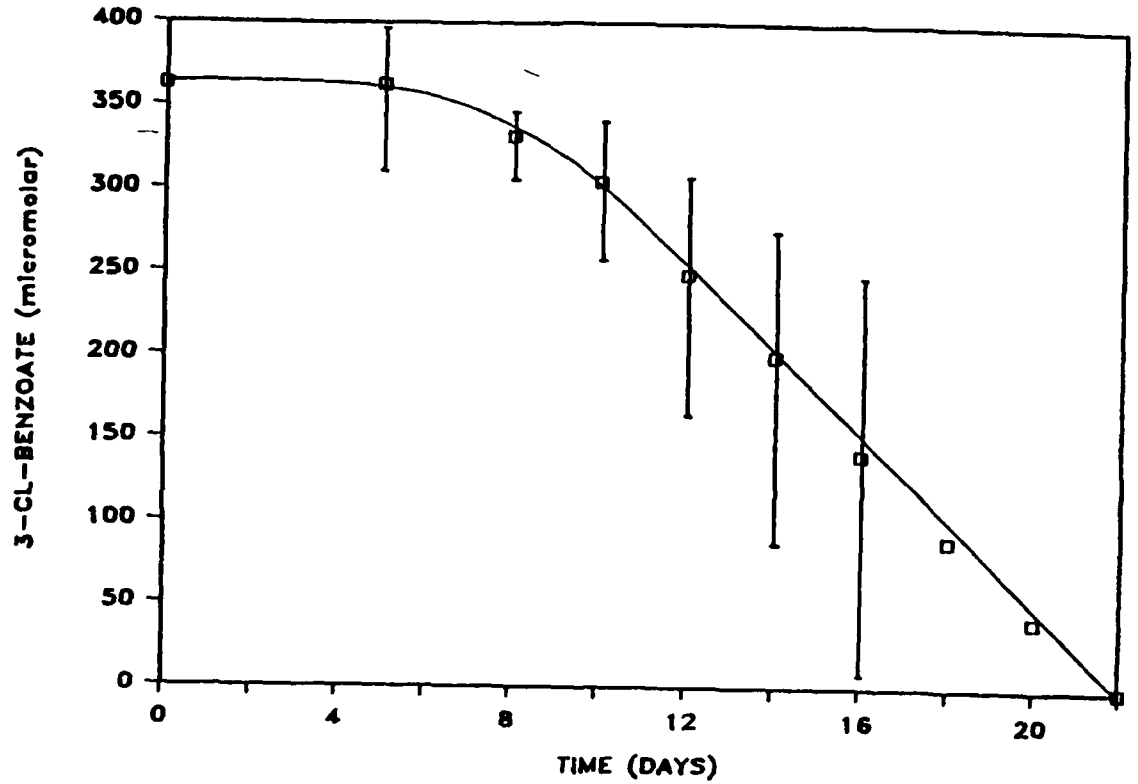

Figure 15. The Dehalogenation of 3-Chlorobenzoate as Influenced by the Batch of Rumen Fluid. Error Bars Indicate the Range of Values for Five Batches. The Mean Time for the Complete Disappearance of 3-Cl-BZ was 22 $\pm$ 5 Days.

(0.63-0.74 μmol l^{-1} h^{-1}) by either reduction in concentration. In similar experiments the concentration of salts (Fe++, MG++, Ca++) was increased 10-fold but no consistent effect on dehalogenation was noted.

No dehalogenation was observed in cultures of rumen fluid minus pyruvate (20.0) or pyruvate minus rumen fluid (0.2) when inoculated with thiosulfate grown cells that had been centrifuged, washed, and concentrated. These experiments show that rumen fluid was critical to dehalogenation and suggest that the reductive process is not related directly to carbon, nitrogen or salt concentration.

4. Dehalogenation in the Presence of Hydrogen

Because the dehalogenation results in replacement of the halogen with hydrogen, the role of H_2 in dehalogenation was examined. Experiments comparing headspace gas composition -- 90:20 mixtures of N_2:CO_2 and H_2:CO_2 -- indicated that hydrogen inhibited dehalogenation. In 25 days 400 μM 3-Cl-BZ was totally dehalogenated under N_2:CO_2, whereas only 50 percent was dehalogenated in the presence of H_2:CO_2 at 0.05 and 0.1 MPa. In other experiments with shaken cultures of 20.2 medium, the dehalogenation rate was 1.21 μmol l^{-1} h^{-1} under N_2:CO_2 but only 0.63 μmol l^{-1} h^{-1} under H_2:CO_2 at 0.1 MPa. Thus, rates of dehalogenation were decreased by as much as 50 percent in the presence of 80 percent H_2 and 20 percent CO_2 as compared to N_2:CO_2 regardless of experimental conditions.

Because strain DCB-1 consumed H_2 (Section III), we examined the relationship of hydrogen consumption to inhibition of dehalogenation. A direct relationship between dehalogenation and hydrogen consumption was found regardless of the initial H_2 concentration (Figure 16). At all three H_2 concentrations initiation of dehalogenation coincided with the termination of hydrogen consumption. Hence, active H_2 consumption appears to divert the cells' metabolic activity for dehalogenation which then begins once H_2 flow ceases.

5. Substrate Range and Rate of Dehalogenation

Other halogenated benzoates served as substrates for dehalogenation, but the dehalogenation activity was confined to the meta position (Table 9). When the activity is expressed per mg protein, the rates were in the same order of magnitude as those rates in the 3-Cl-benzoate enrichment (Reference 16) and the activity among chemicals was similar.

Several nonbenzoate aromatic compounds were also tested: 2-Cl, 3-Cl, and 4-chlorophenol; 2-Cl- and 4-chlorophenoxyacetic acid; 2-Cl, 3-Cl-, and 4-chloroaniline; 3-chloroacetophenone; the three isomers of chlorobenzonitrile; and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Also the other two isomers (ortho and para) of chloro-, bromo-, and iodobenzoate and 3-F- and 4-fluorobenzoate were examined. Of the compounds tested the benzonitriles were the most inhibitory to growth and the most difficult to analyze. At 400 μM concentrations none of these aromatic compounds was significantly dehalogenated as determined by substrate disappearance. Whereas approximately 20 percent of the 2,4,5-T disappeared over 30 days, no products could be found.

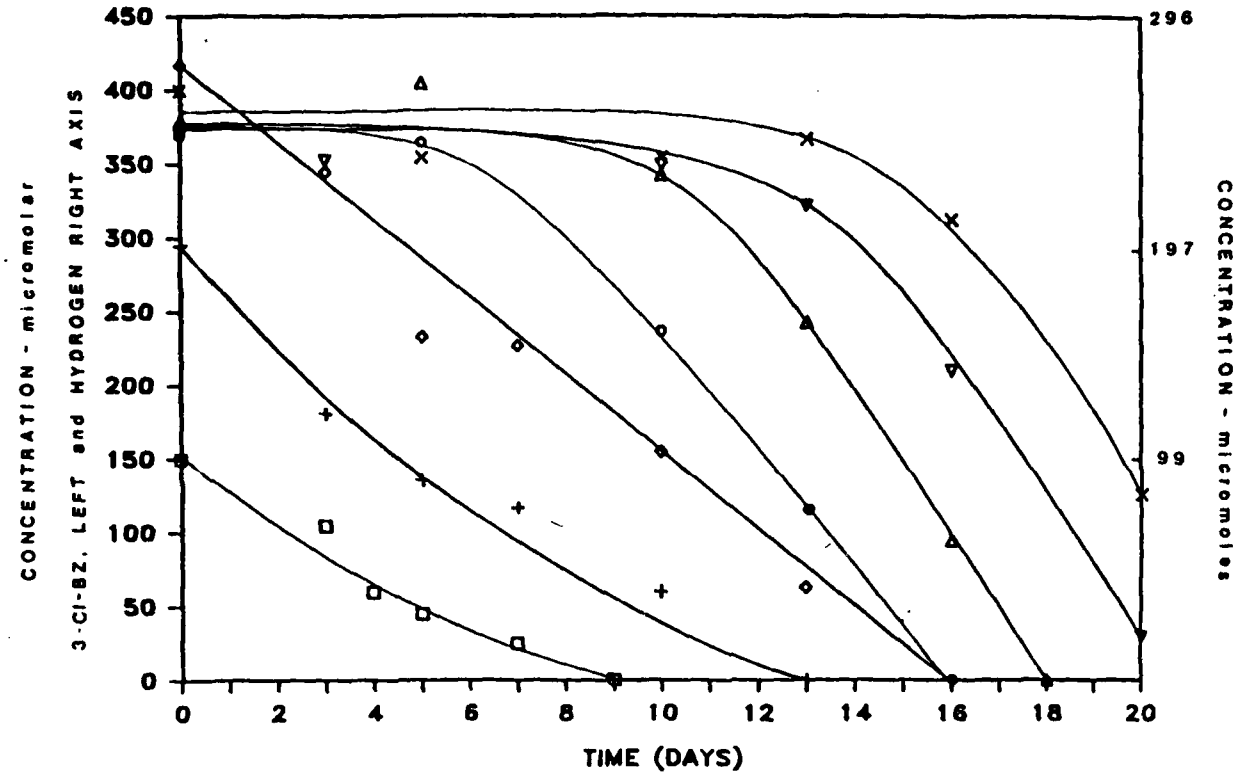

Figure 16. The Correlation of Hydrogen Consumption with Dehalogenation of 3-Chlorobenzoate in 20.2 Medium. SYMBOLS: Squares, Crosses and Diamonds Represent Consumption of 99, 197, and 296 μM of Hydrogen Respectively; Circles, Triangles, Inverted Triangles, and X's Represent Dehalogenation of 3-Cl-BZ in the Presence of 0.0, 99, 197, and 296 μM Hydrogen, Respectively.

TABLE 9. EFFECT OF RUMEN FLUID, TRYPTICASE AND OTHER GROWTH FACTORS ON THE DEHALOGENATION OF 3-Cl-BZ IN THE PRESENCE OF 0.2 PERCENT PYRUVATE AND BASAL SALTS[a].

Growth factor	Order of lag[b]	Dehalogenation rate (μmol l^{-1} h^{-1})	Growth
20 percent rumen fluid + 0.1 percent trypticase	1	1.09	++++
20 percent rumen fluid + 0.1 percent casamiumo acid	1	1.04	++++
20 percent rumen fluid[c]	2	0.93	++++
10 percent rumen fluid	2	0.92	++++
5 percent rumen fluid	3	0.60	+++
1 percent rumen fluid	4	0.60	++
20 percent rumen fluid[c]	1	1.43	++++
1 percent rumen fluid +			
0.001 percent trypticase	2	0.81	+++
0.001 percent trypticase	3	0.07	+
0.01 trypticase	3	0.78	++
0.1 percent trypticase	3	0.27	+++

[a]Data represent mean of two experiments; Three serum bottles per experiment.
[b]Numerical value indicates the relative length of time (days) before dehalogenation started; 1 represents the shortest lag.
[c]Data below the line were from a different batch of rumen fluid.

TABLE 10. DEHALOGENATION RATE OF VARIOUS HALOGENATED BENZOATES (BZ) BY DCB-1 GROWN ON 20 PERCENT RUMEN FLUID AND 0.2 PERCENT PYRUVATE.

Substrates (400 μM)	Product	Dehalogenation rate[a] (μmol h^{-1} mg^{-1} protein)
3-F-BZ	none	0
3-Cl-BZ	BZ	0.093
3-Br-BZ	BZ	0.063
3-I-BZ	BZ	0.075
3,5-diCl-BZ	3-Cl-BZ	0.107
3,4-diCl-BZ	4-Cl-BZ	0.118
2,5-diCl-BZ	2-Cl-BZ	0.074
2,4-diCl-BZ	none	0
2,6-diCl-BZ	none	0
2.3,6-triCl-BZ	2,6-diCl-BZ	0.024
5-Br-2-Cl-BZ	2-Cl-BZ	0.154
4-NH_2,3,5-diCl-BZ	4-NH_2-3-Cl-BZ	0.098

[a]Mean of triplicate experimental bottles.

6. Reductive Removal of Substituents Other than Halogens

If the reductive process could be expressed on substituents more typically found in the natural environment, a greater understanding of the causative mechanism for dehalogenation might be possible. The organism did not dehydroxylate, demethylate, or deaminate the corresponding meta forms of benzoate, but DCB-1 did demethoxylate (or demethylate) 3-methoxy- (Figure 17) and 4-methoxybenzoate (not shown) with the stoichiometric accumulation of the corresponding hydroxybenzoate product. This occurred in the cultures containing 10 mM thiosulfate and basal salts, but lacking both rumen fluid and pyruvate. The reaction increased only slightly when pyruvate was added.

When 3-Cl-BZ at 300 μM and 3-OCH_3-BZ at 1400 μM were present in the same serum bottles containing thiosulfate at 20 mM, no dehalogenation occurred in 10 days even though the conversion of 3-OCH_3-BZ to 3-OH-BZ was complete by this time. Because thiosulfate strongly inhibits dehalogenation but not demethoxylation, the reductive mechanisms are probably different.

7. Induction of Dehalogenation by 3-Cl-BZ

The role of halogenated benzoates in inducing dehalogenation activity was examined. A comparison of dehalogenation at high and low concentrations of 3-Cl-BZ and 4-NH_2-3,5-diCl-BZ indicates that the compounds induced the dehalogenating activity (Figure 18). The implication is that 3-Cl-BZ induced activity at all concentrations, but 4-NH_2-3,5-diCl-BZ was an inducer only at high concentrations. The inability of 4-NH_2-3,5-diCl-BZ to induce activity at 20 μM is similar to the threshold value noted for the same compound during studies on sediment dehalogenation.

The purpose of these experiments was to define those factors that influence dehalogenation, to describe the range of compounds dehalogenated, and to assess if the reductive process occurred on any other substituent. Dehalogenation is biologically catalyzed since: (1) no reaction occurred in autoclaved cultures plus or minus hydrogen, (2) chloramphenicol and sulfanilamide prevented the reaction in cells suspended in 20.2 medium, (3) chloramphenicol stopped the reaction, (4) the reaction occurred in cultures grown from cells that had not been grown in the presence of 3-chlorobenzoate for 2 years, (5) the reaction only occurred at temperatures that allowed growth, (6) oxygen stopped the reaction, and (7) the reaction was independent of certain nutritional factors in the medium. The variability in dehalogenation by DCB-1 grown in various batches of rumen fluid or concentrations of trypticase and the lack of dehalogenation in 20.0 or 0.2 media all support the influence of nutrition on dehalogenation.

Because growth occurred with thiosulfate without concurrent dehalogenation, we showed that the sulfidogen and the dehalogenating organism were the same organism. First, cells grown as thiosulfate or fumarate reducers, centrifuged and resuspended in fresh medium, dehalogenated 3-chlorobenzoate. Second, organisms grown in thiosulfate, containing roll tubes, when picked, and transferred to 20.2 medium minus the electron acceptor grew and dehalogenated 3-chlorobenzoate. Finally, thiosulfate and fumarate grown cells maintained the unique collar characteristic of DCB-1 organism (Reference 59).

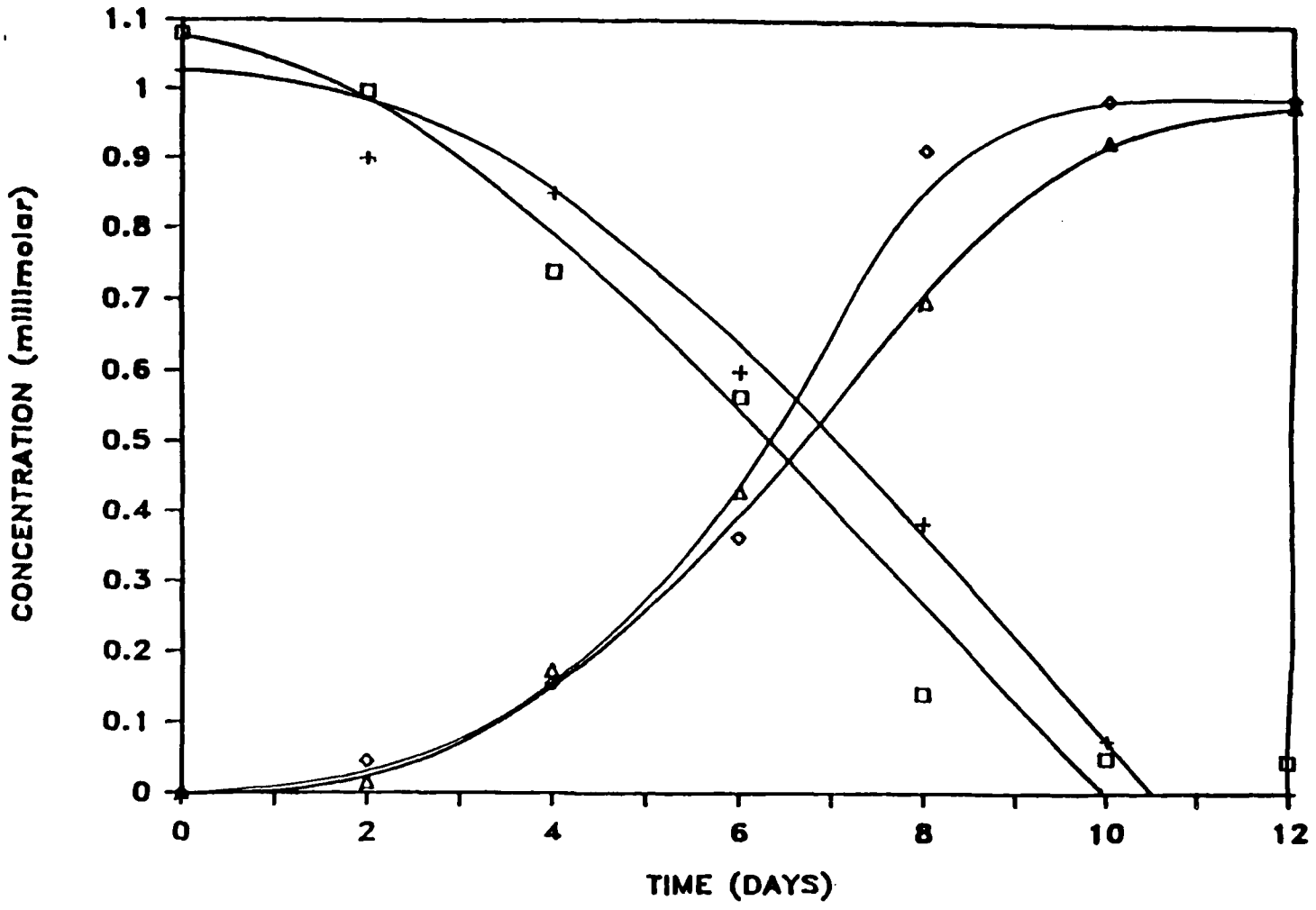

Figure 17. Demethoxylation of 3-methoxybenzoate to 3-hydroxybenzoate in the presence of thiosulfate plus and minus pyruvate. SYMBOLS: diamonds, 3-OCH_3-BZ minus pyruvate; squares 3-OCH_3-BZ plus pyruvate; triangles 3-OH-BZ minus pyruvate; crosses 3-OH-BZ plus pyruvate.

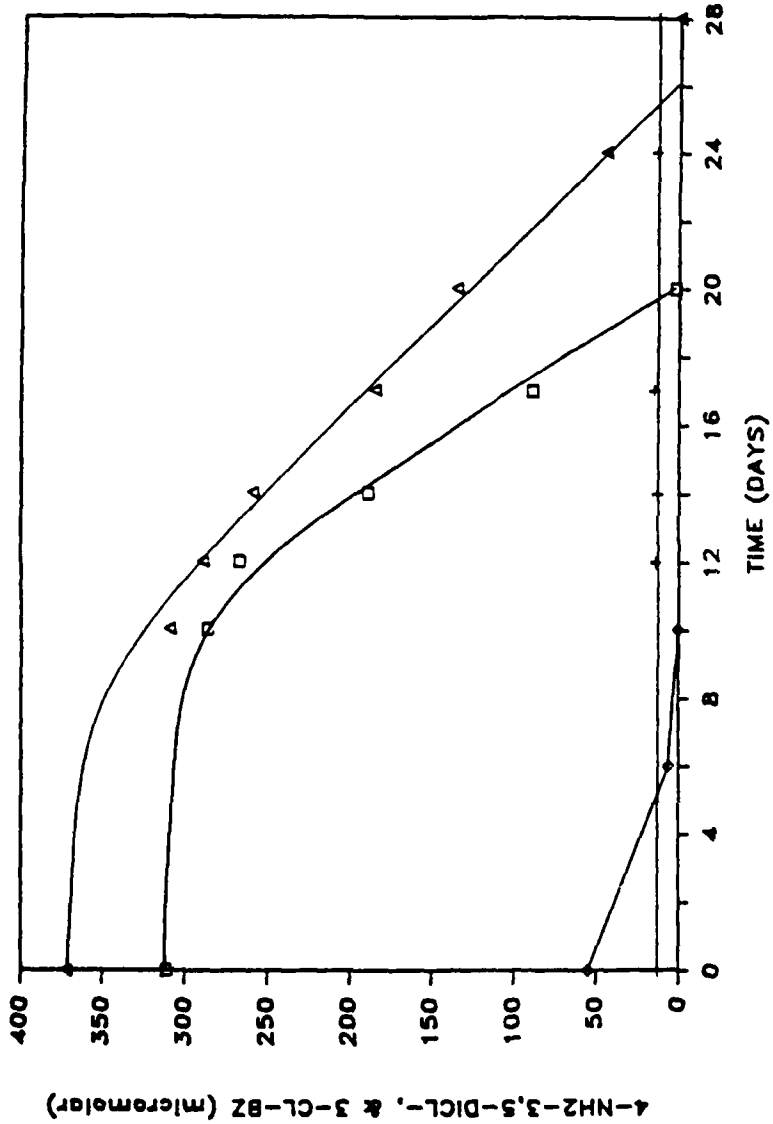

Figure 18. Dehalogenation of 3-Chlorobenzoate and 4-Amino, 3,5-Dichlorobenzoate at Two Different Concentrations. SYMBOLS: Triangles and Diamonds Represent 3-Cl-BZ; Crosses and Squares Represent $4NH_2$-3,5-diCl-BZ.

The effect of electron acceptors suggests that two different pathways are involved in growth: (1) one that yields dehalogenation and (2) one where the mechanism for dehalogenation is shut down. Even though thiosulfate stopped or prevented dehalogenation at high concentrations, its influence was not directly related to the electron accepting ability, because nitrate and fumarate did not inhibit. Fumarate, sulfite and thiosulfate were the only electron acceptors tried that supported growth of DCB-1 on pyruvate with no added nutritional supplement. However, good dehalogenation occurred only in the presence of fumarate (Section III). These results may be consistent with 3-Cl-BZ acting as an electron acceptor because NO_3 and SO_4 would not support growth, and the reduction of fumarate involves a different cytochrome chain than does the reduction of SO_3= or S_2O_3=. Although the actual mechanism of dehalogenation is unknown, the differential inhibition by the various electron acceptors provides an important clue for further investigation.

Hydrogen is also an important factor in dehalogenation and may also provide a clue to the mechanism. Hydrogen inhibited dehalogenation which suggested that the hydrogen used to replace the chlorine atom is not the direct product of hydrogenase activity. Furthermore, electron flow from H_2 use seems to divert the normal flow of electrons from the reductive dehalogenation. Although H_2 is used by DCB-1, it is not produced by this strain. Desulfovibrio sp. are known to produce (References 35, 60) and consume (References 61, 46) hydrogen, and this has been explained by suggesting the presence of reversible hydrogenase (Reference 62) or the development of metabolically different hydrogenases located in two different regions of the cell (References 19, 63). Cytoplasmic hydrogenases are responsible for hydrogen production whereas the periplasmic types consume hydrogen (References 19, 63). The physiological basis for the dual hydrogen metabolism, however, has produced different theories of which hydrogen cycling (Reference 19) and trace hydrogen transformation (Reference 58) are the most prominent. Because DCB-1 produces no H_2, it may lack a cytoplasmic hydrogenase. Only by defining the mechanism of dehalogenation can the roles of hydrogen and electron acceptor be elucidated.

The substrate range at this time is limited to the benzoates. As stated above, compounds with functional groups other than COOH have been tested in trypticase, 20.2 media, with electron acceptors, without electron acceptors, added to culture actively dehalogenating 3-chlorobenzoate (2 compounds), and in media inoculated from cultures that had just previously dehalogenated 3-chlorobenzoate. This apparent enzyme specificity further indicates that the dehalogenation is a microbially catalyzed process. That the organism did not dehalogenate 2,4,5-T is surprising because the parent sludge enrichment did. Perhaps it takes longer to develop the enzymes than batch culture will allow or a nutrient dependence remains unsatisfied, or another organism exists in the enrichment (Reference 58). In all cases the rates of dehalogenation are slow (16-28 days for 400 μm). Several compounds, e.g. benzonitriles and 2,4,5-T are toxic but growth eventually develops in their presence. Concentrated inocula seem to yield faster dehalogenation rates than more dilute inocula.

Dehalogenation is important due to the quantity of halogenated compounds now found in nature. Whereas naturally occurring halogenated compounds exist which could serve as natural substrates (References 1, 65) a majority are probably marine in origin (Reference 66). A more likely explanation for dehalogenation is that enzymes responsible for cleaving other substituents may also fortuitously cleave halogens. Aromatic ring changes include the reductive alteration or removal of ring substituents including dehydroxylation (References 67, 68, 69), demethylation (References 69, 70), decarboxylation (References 69, 70, 71), and the conversion of a phenoxy alkanoate to a hydroxy substituent (Reference 19). These reductive mechanisms are strongly influenced by other ring substituents (Reference 70). DCB-1 reductively converts 3-OCH_3-BZ to 3-OH-BZ, indicating that its reductive capacity is not confined to the removal of halogens. Because this organism is capable of growth via this reductive process, demethoxylation might explain dehalogenation. However, because 3-Cl-BZ in the presence of thiosulfate is not dehalogenated simultaneously in cultures where demethoxylation is occurring, the enzymes and processes are apparently different.

In order to expand the range of dehalogenation two approaches can be pursued: (1) mutation of the pure culture, or (2) enrichment adaptation. Beyond exploring the relation to demethyoxylation, expanding the reactivity in pure culture is limited to chance until the mechanism is described. Due to its wider range of activity and methanogenic nature (Reference 58), the enrichment maybe the more logical environment to study. By using varying concentrations of methoxylated and halogenated compounds the enrichment may be slowly adapted to a wider base of activity. The parent enrichment (Reference 58) displays some para and ortho dehalogenating ability on benzoates as well as on 2,4,5-T (Reference 16) yet beyond meta-halogenated and meta-methoxylated compounds, the pure culture only supports para demethoxylation. Perhaps the right combination of factors is lacking for full reductive expression.

We have described the basic dehalogenating capacity of this organism under defined conditions in pure culture. Many aspects of the organism's physiology remain unanswered yet research directed towards the organism's metabolism will be rewarding. In particular several projects should be considered: (1) the relationship between hydrogen metabolism and carbon pathways, (2) the roles of electron acceptors and hydrogen, (3) a comparison of this organism's hydrogen metabolism with that of other known sulfidogens, (4) the relationship (if any) between reductive dehalogenation and demethyoxylation, and (5) expanded attempts to broaden the range of substrates dehalogenated.

Finally, dehalogenation is characterized by several distinctive features that are deserving of more study. First, determining the natural substrate will aid in understanding the mechanism of dehalogenation and the influence of dehalogenation on the organism. Second, the dehalogenating capacity exists even after years of nonexposure to 3-chlorobenzoate which suggests that the process is chromosomal and not plasmid controlled. Finally, the organism may have threshold values below which dehalogenation will not occur.

SECTION V

COMPLETE REDUCTIVE DECHLORINATION AND MINERALIZATION OF PENTACHLOROPHENOL BY ANAEROBIC MICROORGANISMS

A. RATIONALE AND APPROACH

In previous work we had shown that most of the mono and dichlorinated phenols could be dechlorinated but that the positions attached were only those in the meta, ortho, and para enrichment substrate. Collectively, however, it appeared that a combination of the three enriched populations could degrade pentachlorophenol. This approach should allow us to evaluate the overall goal of this project which was to assess whether aromatic compounds could be dechlorinated if adjacent carbon atoms also were substituted (in this case with Cl or OH). The section demonstrates that all chlorine can be removed and the majority of the ring carbon is mineralized as well.

B. MATERIALS AND METHODS

Anaerobically digested municipal sewage sludge was taken from a primary digester at the wastewater treatment plant in Jackson, Michigan. The plant receives about 40 percent of its wastewater from industrial sources and the rest is residential. The retention time in the primary anaerobic digester is approximately 13 days. Typically the sludge contains about 40 grams solids kg^{-1} sludge with a total N content of about 40 grams N kg^{-1} dry sludge (J. St. Andre, R.A. Greene Wastewater Treatment Plant, 1985; personal communication).

The sludge was collected from the digester and placed in 4-liter glass bottles and transported to the laboratory where the headspace of each bottle was thoroughly flushed with an 0_2 free gas mixture (80 percent N_2 - 20 percent CO_2) using a Hungate apparatus (References 72, 73). Separate bottles of sludge have been maintained for more than 2 years with chlorophenol as the only added carbon or energy source. Chlorophenol was added weekly from 6000 μg mL^{-1} aqueous stock solutions to give chlorophenol concentrations of approximately 25 μg mL^{-1}. Batches of sludge have also been maintained with simultaneous feedings of all three monochlorophenol isomers. This sludge received approximately 10 μg mL^{-1} of each monochlorophenol isomer per week from the aqueous stock solutions. Chlorophenol concentrations in the acclimated sludges were checked each week to prevent accumulation of chlorophenol due to excess feeding.

The studies with PCP and pentabromophenol were conducted with three sludges: (a) the individual monochlorophenol acclimated sludges; (b) a mixture of equal volumes of the three monochloropherol sludges; and (c) sludge acclimated simultaneously to the three monochlorophenol isomers. In each case the sludges were dispensed with a glass pipette in duplicate into 160 mL serum bottles which had been thoroughly flushed with an 0_2 free gas mixture. For items (a) and (c) above, 75 mL of sludge was transferred; for item (b), 25 mL of each monochlorophenol sludge was transferred to the serum bottle. PCP (40 μM, 11 μg/mL) was added to the bottles from a concentrated (15 percent)

ethanol stock solution using a microliter syringe. After flushing the headspace for an additional 2-3 minutes the bottles were sealed with butyl rubber stoppers and aluminum crimp seals. Incubation was in the dark at 37°C without shaking, and samples (1-2 mL) were withdrawn daily using a disposable syringe and frozen until the time of analysis. Every 4 days duplicate samples were taken so that an immediate analysis could be made to determine whether refeeding was necessary. Sterile controls were provided by autoclaving twice on successive days. The individual acclimated sludges received four additions of PCP over 30 days. The acclimated sludge mixture and the simultaneously acclimated sludge received five and three PCP additions, respectively in 18 days.

To extract the chlorophenols the 1.0 mL sample was mixed with 0.5 mL acetonitrile on a vortex mixer, centrifuged for 10 minutes at 10,000 x gram, and filtered through a 0.45 μm membrane (Millipore type HVLP) filter. This procedure resulted in recoveries of 85 percent or better when sludge was spiked with 25 μg mL^{-1} chlorophenol. The concentration of chlorophenols was determined using a Waters high-performance liquid chromatography (HPLC) system consisting of the following components: models 6000A and M45 pumps, model 720 system controller, and model 480 variable wavelength UV detector. The chlorophenols were detected at their absorbance maxima, ranging from 280 to 300.5 nm. The detection limit was 0.5-0.1 μg mL^{-1}. The samples were injected onto an ODS column (4.6 x 25 cm) using a Rheodyne 7125 sample injector with a 20 μL loop. Peak area values were obtained from a Waters data module integrator. The mobile phase consisted of acetonitrile and 5 percent aqueous acetic acid in proportions which were varied to give retention times of 4 to 8 minutes at a flow rate of 2.0 mL min^{-1}. The mobile 1 phase composition ranged from 70/30 to 45/55 CH_3CN/5 percent aqueous CH_3COOH.

The experiments with ^{14}C-PCP were conducted in the same type of serum bottle incubation system described above. Approximately 4 μCi of ^{14}C-PCP was added from an ethanol solution with a microliter syringe to each bottle. The experiment was performed with the mixture of acclimated sludges. Half the bottles were fed monochlorophenols (approximately 2 μg mL^{-1} $week^{-1}$ of each isomer) and the other half contained only ^{14}C-PCP. At the end of incubation the liquid in each bottle was acified wtih H_3PO_4 and the headspace was checked for $^{14}CO_2$. The headspace CO_2 was trapped by flushing through a series of five scintillation vials containing 10 mL of 0.1 N NaOH. A sample of trapping solution was removed from each vial and added to scintillation cocktail for the determination of radioactivity. That $^{14}CO_2$ was the source of radioactivity in the trapping solution was verified by precipitation of CO_2 with $BaCl_2$ and counting an aliquot of the remaining liquid. In all cases the radioactivity remaining after precipitation of the CO_2 was at background level. The recovery of $^{14}CO_2$ in sludge spiked with ($^{14}CO_2$) bicarbonate was 78 percent. The values reported have been adjusted for this recovery.

After the headspace from the serum bottles was flushed, the sludge was centrifuged at 26,000 x gram for 20 minutes, to separate the solids from the acidified aqueous phase. The solids were then extracted twice with methanol and twice with ethyl acetate and the ^{14}C in these extracts was determined.

The ^{14}C remaining in the air-dried sludge solids was collected as $^{14}CO_2$ after the solids were combusted in the solids in a Harvey Biological Materials Oxidizer (model OX200). The ^{14}C-radioactivity was determined with a Beckman model 5000 LSC using external standard quench correction.

All chlorophenols were obtained from Aldrich Chemical Co. (Milwaukee WI) and were used without further purification; ^{14}C-PCP (10.57 mCi $mmol^{-1}$), uniformly ring labelled, was purchased from Pathfinder Laboratories (St. Louis MO) and had a radiochemical purity of > 98 percent. Pentabromophenol (PBP) was purchased from Ultra Scientific (Hope RI).

C. RESULTS AND DISCUSSION

Pentachlorophenol (10 μg mL^{-1}) was degraded in each of the individual monochlorophenol acclimated sludges (Figure 19). Degradation occurred most rapidly in the 2-CP acclimated sludge (Figure 19a), in which the PCP was degraded within 3 days. PCP degradation in 3- and 4-CP acclimated sludges (Figures 19b and 19c) was considerably slower, requiring 12 and 9 days, respectively, for the complete disappearance of PCP. No PCP degradation occurred in autoclaved controls. The time-course for monochlorophenol degradation is also given in Figure 19, which show that 2-CP was degraded more rapidly than the other monochlorophenol isomers. These data together with our previous results on dechlorination of chlorophenols (References 5, 19, 21) suggest that Cl substituents ortho to the phenolic OH group are removed more rapidly than Cl in the meta and para positions. Thus, removal of the ortho-Cl substituents of PCP by the 2-CP acclimated sludge probably occurred at a faster rate than removal of the meta or para-Cl substituents of PCP by the 3-CP or 4-CP sludges. Differences in cell numbers between the three acclimated sludges may have also contributed to the different rates.

The individual CP acclimated cultures dechlorinated PCP, principally, but not exclusively at the position corresponding to the monochlorophenol to which they were acclimated. Figure 20 indicates the products of PCP dechlorination which accumulated in the acclimated sludges. In 2-CP acclimated sludge 3,4,5-trichlorophenol (3,4,5-TCP) and 3,5-dichlorophenol (3,5-DCP) accumulated in a ratio of 2:1; in 3-CP acclimated sludge 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP) and 2,4,6-TCP accumulated in a 1:2 ratio; and in the 4-CP acclimated sludge 2,3,5,6-TeCP and 2,3,5-TCP accumulated in a ratio of 3:1. These data show that the lower chlorinated phenols (TeCPs and TCPs) were less subject to dechlorination reactions than PCP, and thus tended to accumulate. It was also apparent that the dechlorinating populations present in the three acclimated sludges were qualitatively different. The pattern of dechlorination in the acclimated sludges was similar to that obtained in an earlier study (Reference 21) in which monochlorophenol acclimated sludges were tested for activity on different chlorophenol isomers. In the earlier study the 2-CP acclimated sludges were able to dechlorinate 4-CP. The specificity of the populations with regard to site of dechlorination argues strongly for an enzymatic event.

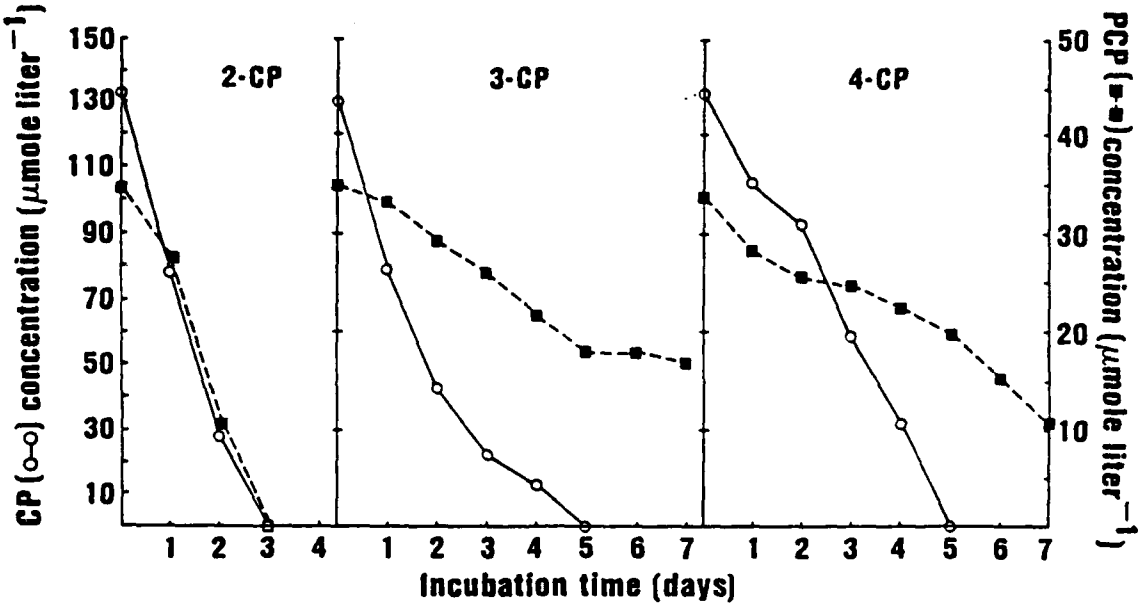

Figure 19. Degradation of Monochlorophenols and Pentachlorophenol in Sludges Acclimated to 2-, 3-, and 4-Chlorophenol (CP).

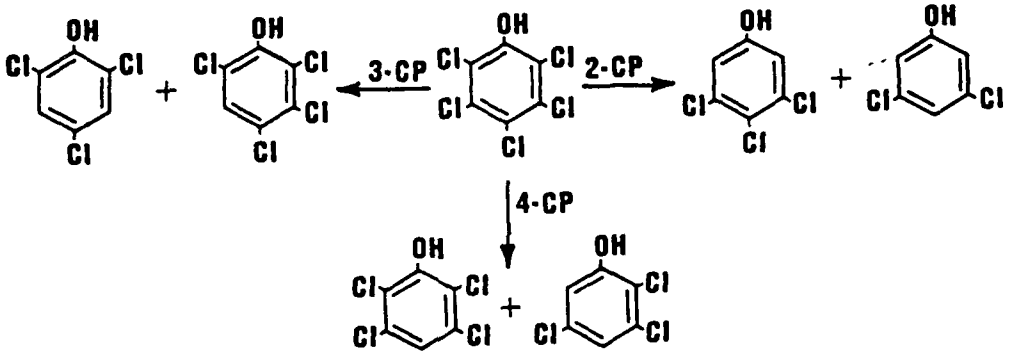

Figure 20. Products Resulting from the Dechlorination of PCP in Sludges Acclimated to 2-, 3-, or 4-Chlorophenol.

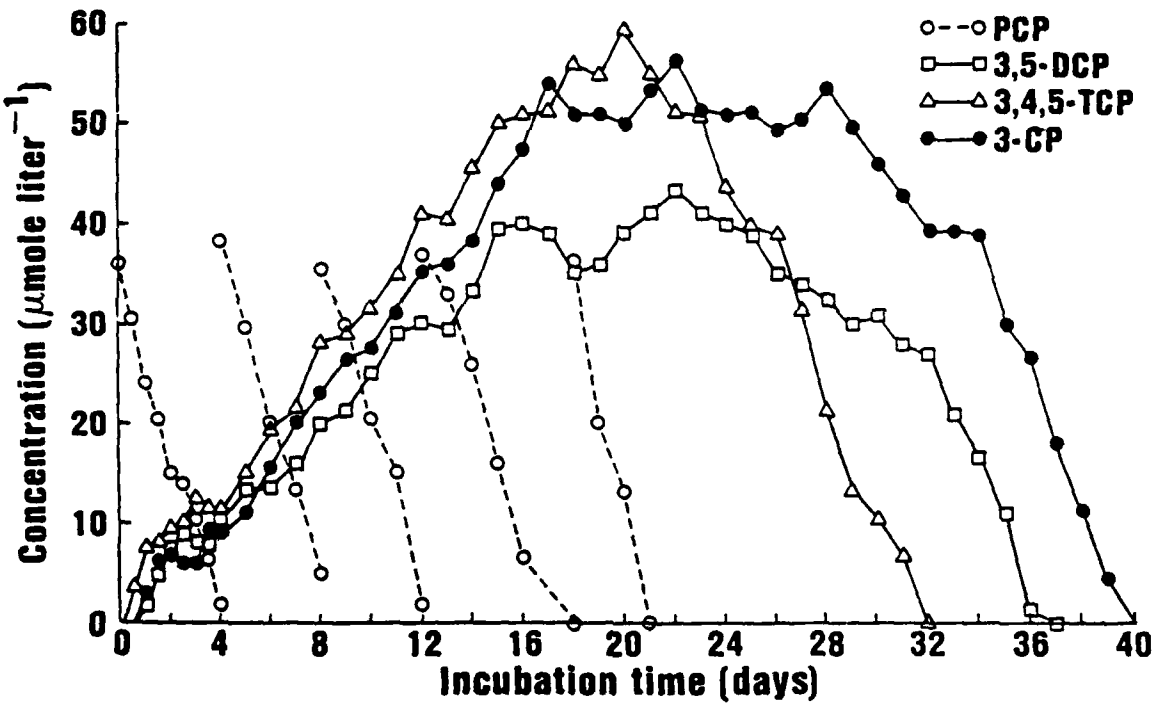

Figure 21. PCP Degradation in a Mixture of 2-, 3- and 4-Chlorophenol Acclimated Sludges, Showing Sequential Appearance and Disappearance of Dechlorination Products.

The three monochlorophenol acclimated sludges, if considered together, were able to dechlorinate at all positions on the aromatic ring. The next step toward our objective of complete PCP degradation was to combine the three dechlorinating activities and determine if PCP was completely dechlorinated. Figure 21 shows the results of the experiment where equal volumes of 2-, 3-, and 4-CP sludges were mixed and given five PCP additions over a period of 18 days. Each time PCP was added (40 μM, 11 μg/mL), it was degraded within 3-6 days, with the gradual accumulation of 3,4,5-TCP, 3,5-DCP, and 3-CP. The maximum accumulation of dechlorinated products (21st day) was 75 percent of the PCP added. No PCP was present after the 21st day, and the concentration of accumulated products gradually declined until, by the 40th day, no chlorophenols could be detected. The products disappeared in the expected sequence, with 3,4,5-TCP giving way to 3,5-DCP, and finally 3-CP. In general, accumulation of phenol was not observed in the acclimated sludges or enrichments; phenol was apparently consumed as rapidly as it was produced from the final dechlorination step. No PCP degradation occurred in the autoclaved controls.

The complete dechlorination of PCP with this type of accumulation and disappearance of lower chlorinated phenols was also observed in sludge which had been acclimated over a long period to all three of the monochlorophenol isomers simultaneously (results not reported). The same product accumulation and disappearance pattern occurred in the simultaneous acclimation as in the mixed system; the only significant difference was that the rate of PCP degradation was slower in the former case.

These results clearly show the complete reductive dechlorination of PCP through the combined activities of two or three anaerobic chlorophenol-degrading populations. We have proposed a degradation pathway for PCP, based on the sequential appearance and disappearance of 3,4,5-TCP, 3,5-DCP and 3-CP (Figure 22). This pathway appears to result from the relatively higher rate of PCP dechlorination by 2-CP acclimated sludge (Figure 19). This sludge rapidly removes Cl from the 2 and 6 positions of PCP to give 3,4,5-TCP (Figure 20). The para-Cl was then removed by populations present from the 2-CP and/or the 4-CP acclimated sludges which have been shown to dechlorinate this position (Figure 20 and Reference 21). 3,5-DCP and 3-CP were likely dechlorinated by the 3-CP acclimated sludge. Previous results (Reference 21) have shown that the 3-CP sludge was cross-acclimated to the degradation of 3,5-DCP.

The ultimate fate of the PCP carbon was investigated by adding ^{14}C-PCP to the CP-acclimated sludge mixture. After a 2-month incubation duplicate bottles were analyzed for the ^{14}C in gas, aqueous, and solid phases, and in extracts of the solids. The results are summarized in Table 11. The data in the two columns are for a sludge mixture which was given weekly additions of the three monochlorophenols and a mixture which was incubated without additions. The reason for comparing the two treatments was to determine if the presence of chlorophenols would affect the dechlorination and mineralization of PCP. The data show that the presence of chlorophenols resulted in less mineralization of PCP, as indicated by lower amounts of gaseous ^{14}C and higher aqueous and extractable levels of ^{14}C. When the

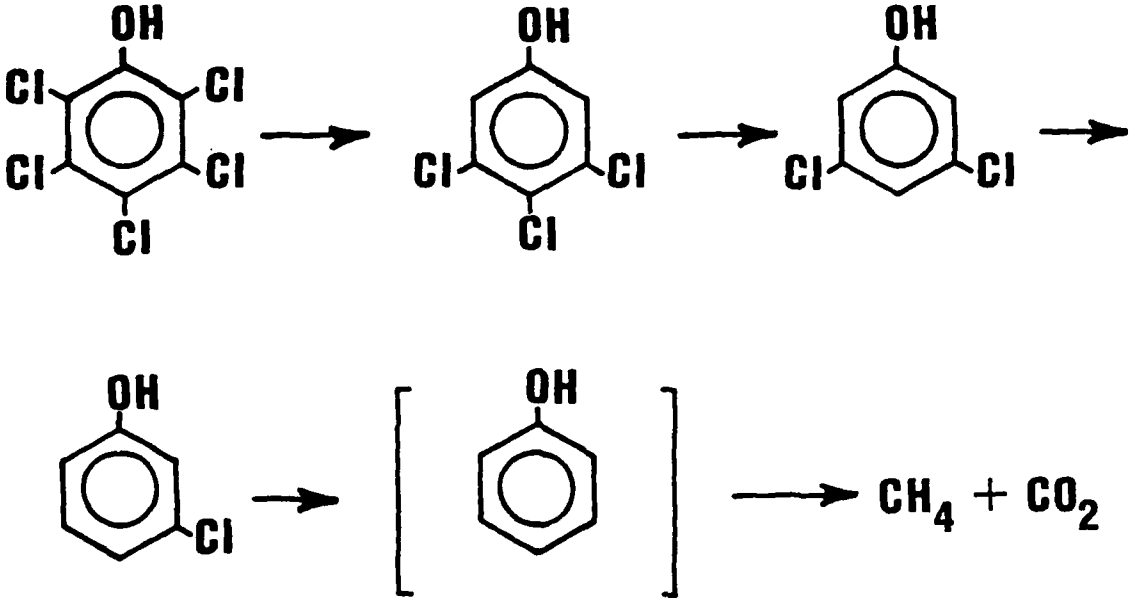

Figure 22. Pathway of PCP Degradation in the Mixture of CP Acclimated Sludges.

TABLE 11. DATA SHOWING MINERALIZATION OF ^{14}C-PCP.

Products	Percentage of added ^{14}C in fraction	
	Amended with 2-, 3-, and 4-CP	Not amended
CO_2	22.9	27.4
CH_4[a]	32.0	38.3
Total ^{14}C in gas phase	54.9	65.7
Aqueous phase	13.7	10.9
Methanol extract of solids	16.9	12.3
Ethyl acetate extract of solids	5.5	4.8
Extracted solids	4.3	4.1
Total ^{14}C-recovery	95.3	97.8

[a]Calculated from the measured CO_2 according to equation: phenol -----> $2.5CO_2 + 3.5CH_4$ (Healy and Young, 1978).

amounts of ^{14}C trappad as $^{14}CO_2$ were combined with the calculated amount of $^{14}CH_4$ produced (according to the stoichiometry of phenol degradation in methanogenic consortia, (Reference 74), the total carbon mineralized was substantial, 54.9 percent and 65.7 percent, respectively, for the fed and not fed sludges. This difference is quite small and may be due, in part, to a small isotopic dilution effect resulting from the presence of the added CP's. The total recovery of the added ^{14}C was greater than 95 percent for all bottles analyzed.

The degradation of technical grade PCP and reagent grade PCP were compared (Figure 23). No significant difference in the rate of disappearance of the two forms was apparent until the third PCP addition, at which time technical grade PCP was being degraded more slowly. Technical grade PCP formulations are known to contain a variety of highly toxic contaminants (References 75, 76) not present in reagent grade PCP. The potential contaminants include chlorinated dibenzo-p-dioxins, dibenzofurans, phenoxyphenols and anisoles, and could be responible for the retarded disappearance of technical grade PCP.

The degradation of pentabromophenol (PBP) by the mixed acclimated sludges was also observed (Figure 23). The rate of disappearance of PBP was greater than PCP through all three additions (over 28 days). Thus, it appears that dehalogenation occurred for both chloro- and bromo-aromatic compounds, and that removal of Br substituents may be more facile.

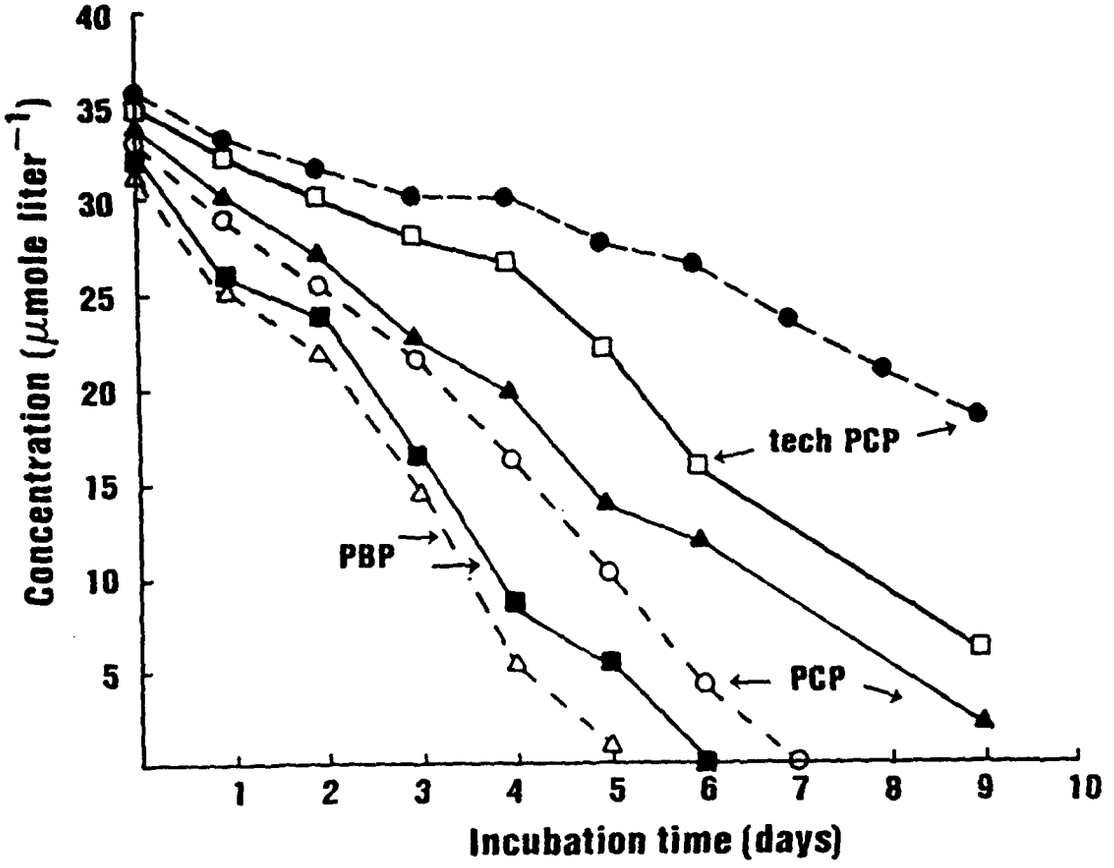

Figure 23. Degradation of Technical Grade PCP, Reagent Grade PCP, and PBP in the Mixture of CP Acclimated sludge. Solid lines represent first addition; dashed lines represent third addition.

SECTION VI

REDUCTIVE DECHLORINATION OF CHLORINATED PHENOLS IN ANAEROBIC UPFLOW BIOREACTORS

A. RATIONALE AND APPROACH

Anaerobic upflow bioreactors have been used as a continual treatment system in order to exploit the enzymatic activities involved in the dechlorination and degradation of chlorophenols. A two-stage reactor system consisting of an anaerobic upflow bioreactor and an aerobic trickling filter was utilized for the treatment of chlorophenolic waste from paper pulp bleaching (References 77, 78, 79). Chlorophenols were removed from the waste stream and mineralization of added pentachlorophenol to CO_2 was observed. The extent of chlorophenol biodegradation due to each system was not determined and methane was not reported as a product. In an anaerobic upflow bioreactor which received readily utilizable carbon sources in addition to chlorophenols Woods (Reference 80) found removal of the ortho- and meta-chlorines from chlorophenols containing two or more chlorines. Para-chlorines were not removed and ring cleavage was not observed.

In this study, we examined the reductive dechlorination of chlorinated aromatic compounds in anaerobic upflow bioreactors using chlorophenols as the sole carbon and energy source. This is the first step towards utilizing these unique dechlorinating activities for large-scale treatment. Specific research objectives were to: (1) determine whether dechlorinating activity can be maintained in anaerobic upflow bioreactors with chlorophenols as the sole carbon and energy source, (2) determine if mineralization of the chlorophenols occurs in the bioreactors, (3) examine the bioreactor for morphologic types and distribution of colonizing bacteria, (4) determine the substrate range of the bioreactor community, and (5) determine the substrate loading capacity of the established bioreactors.

B. MATERIALS AND METHODS

1. Bioreactor Design and Procedures

All experiments were conducted using anaerobic upflow bioreactors as shown in Figure 24. The cylindrical plexiglass reactor was approximately one liter in volume with a height of 12 inches and an inner diameter of 3 inches. The ends of the reactor were cone-shaped and threaded for easy removal. Cajon fittings were used to connect either 1/4-inch I.D. glass or stainless steel tubing to the cylinder. Sampling ports sealed with butyl rubber stoppers were located throughout the length of the bioreactor.

Two areas inside the bioreactor were designed for the containment of the biomass. A fine mesh stainless steel screen located at the bottom of the reactor was designed for the accumulation of a sludge blanket. The center of the bioreactor, designed for the attachment of the biomass, contained approximately 250 mL of 4 mm etched glass beads held in place by an additional screen.

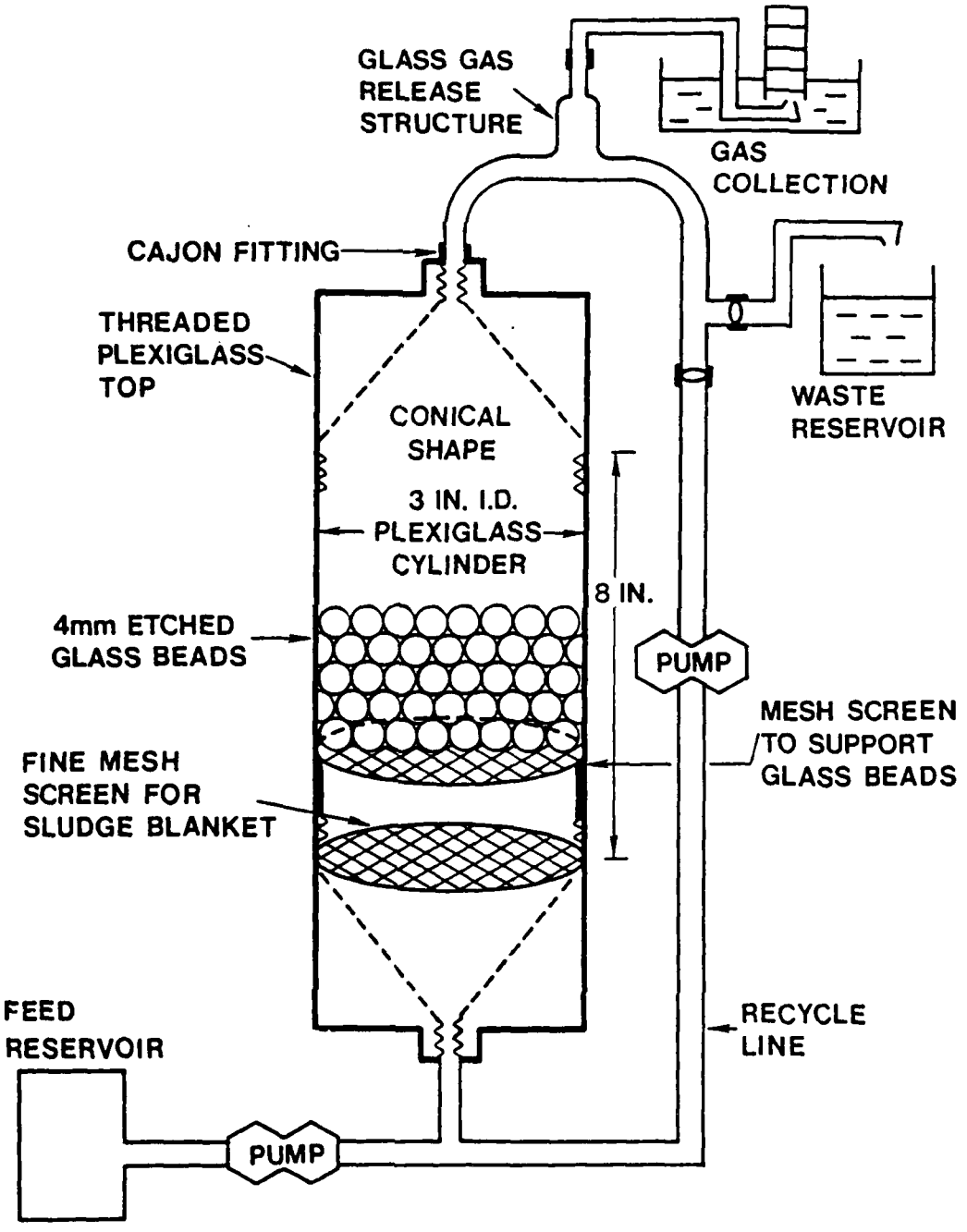

Figure 24. Diagram of Anaerobic Upflow Bioreactor.

Gaseous end products were separated from liquid effluent in a glass gas-release structure attached to the top of the cylindrical bioreactor. Gas was collected in an inverted graduated cylinder placed in an acid-brine solution (2 percent HCl, 10 percent NaCl). The top of the graduated cylinder was fitted with a butyl rubber stopper for gas measurements. Effluent was collected in a 1 liter Erlenmeyer flask from a port originating from the glass gas release structure.

The feed reservoir, a 1 liter Erlenmeyer flask, was completely sealed by a rubber stopper and held in place by plastic tape. Tygon and stainless steel tubing connected the bottom of the feed flask through the stopper to a piston-action pump (Fluid Meter, Inc. RP6 pump equipped with a 1/4-inch ceramic piston pump head). Additional tubing connected the pump to the bottom of the reactor.

A slow continual stream of oxygen-free nitrogen, provided by a tank connected in series to a two-stage regulator, a low pressure regulator (Matheson Model 70) and an adjustable relief valve (Union Carbide Corp. RV5570), was supplied to the feed flask headspace via the rubber stopper. The pressure release valve was set at 1-2 psi to prevent excess pressure build-up in the feed flask. The feed flask, containing anaerobic medium, was replaced as needed.

Heat distribution inside the bioreactor was provided by eight aluminum strips (7.5 inches x 3/4-inch x 1/4-inch) attached to the outside of the column. Column was heated by means of a heat resistor tape wrapped around the outside of the aluminum strips and attached to a variable transformer. The temperature was controlled either by setting the transformer or by connecting the transformer to a temperature controller (YSI Model 64RC, Yellow Springs Instrument Co., Inc.) equipped with a YSI Series 600 thermistor attached to the outside of the column. Temperature was monitored by a thermometer placed inside the bioreactor through a top well sealed by a Cajon fitting. The entire bioreactor was placed inside a wooden box packed with styrofoam for insulation.

The bioreactors were inoculated using strict anaerobic techniques by initially flushing the bioreactors with oxygen-free nitrogen before adding either acclimatsd anaerobic sewage sludge or enrichment cultures. Anaerobic sewage sludge, obtained from a primary anaerobic sewage sludge digestor (Jackson MI), was acclimated for approximately two years by weekly feedings (20 $\mu g\ mL^{-1}$) of chlorophenols. Enrichments were obtained by periodic transfer (25 percent) of acclimated sludge into revised anaerobic mineral medium (RAMM) (Reference 3). Enrichments were maintained by feeding approximately 10-20 $\mu g\ mL^{-1}$ chlorophenols as the sole carbon and energy source every 2 days. Both acclimated sludge and enrichment culture were periodically analyzed for chlorophenol to prevent a toxic accumulation of the substrate.

Bioreactors I and III were initially inoculated with an enrichment that had been degrading meta-chlorophenol for approximately 1 year. The enrichment was obtained by two transfers of 50 percent and 25 percent from acclimated sludge. The bioreactors were completely filled with the enrichment culture.

Bioreactor II was initially inoculated with an equal mixture of three sludges, each acclimated to one of the monochlorophenols. The bioreactor was seeded with a 1:1 mixture of sludge and medium. When activity in Bioreactor II diminished at day 200, the bioreactor was reseeded with 100 mL (10 percent) of a 1:1:1 mixture of sludge acclimated to each of the monochlorophenols.

The bioreactors were continually supplied with medium containing chlorophenol which was added immediately prior to use. Meta-chlorophenol was the sole carbon and energy source for Bioreactors I and III and a 1:4:1 mixture of ortho-: para-: meta-chlorophenol was the sole carbon and energy source for Bioreactor II.

Effluents were collected and measured daily to determine the hydraulic retention time (HRT). Daily samples of the effluent were frozen for later analysis. The average influent and effluent substrate fluxes (mg l^{-1} day $^{-1}$) were calculated from the influent and effluent substrate concentration and the HRT. The substrate conversion efficiencies were calculated from the average influent and effluent substrate concentration.

2. Analytical Procedures

Total volume of gas collected in the inverted cylinder was measured daily and samples of the gas were taken periodically for methane quantification by injecting 0.2 mL of the gas into a Carle gas chromatograph equipped with a Porapak® Q column and a microthermistor detector. Dissolved methane in the effluent was extracted according to the procedure developed by Robinson et al. (Reference 81) for extracting dissolved H_2. Samples of the effluent were taken after separation of gaseous products in the gas release structure (Figure 24), and before exposure to the atmosphere, by piercing the tygon tubing with a 22-gauge needle and slowly withdrawing a sample into a glass 50 cc syringe fitted with a one-way stopcock. A Bunsen absorption coefficient of 0.03046 was used in the calculations (Reference 82).

Chlorophenols were analyzed by high-pressure liquid chromatography (HPLC). Effluent samples were thawed and then filtered through 0.45 μM HA Millipore filters. The monochlorophenols were routinely measured with a Waters HPLC consisting of a model 6000A pump and a model 441 UV absorbance detector set at 280 nm. The sample injection valve (Rheodyne 7125) was fitted with a 20-μL loop. The analytical column was a Waters Radial PAK C18 cartridge held in a RCM-100 Radial Compression Module. Peak areas were measured with a Hewlett Packard 3390A integrator. The trichlorophenols and pentachlorophenol were measured with a Waters HPLC consisting of a model 6000A pump, a model 45 pump, and a model 720 systems controller, coupled with a model 480 Lamba Max variable wavelength UV absorbance detector. The wavelength was set at 300.5 nm for pentachlorophenols and 280.0 nm for trichlorophenols. The analytical column was a uBondapak® C18 column (Waters Assoc.).

Mobile phase for both HPLC systems consisted of a 5 percent aqueous acetic acid-acetonitrile mixture with a flow rate of 2.0 mL min^{-1}. The mobile phase was adjusted to give a retention time of approximately 3.5 minutes for the

monochlorophenols (1:1 mixture); 5 minutes for trichlorophenols (4:6 mixture); and 7 minutes for pentachlorophenol (3:7 mixture). Mixtures of the monochlorophenols were separated by using a mobile phase mixture of 8:2 resulting in retention times of 5.7 minutes.

3. Mineralization Studies

The mineralization of meta-chlorophenol was examined in Bioreactor I by quantitative recovery of the mineralization product, CH_4. The study took place during Days 159 through 202 of operation with an average influent substrate of 10.2 mg l^{-1} day^{-1} and an average HRT of 6.7 days. The substrate conversion efficiency was 100 percent. Both gaseous and dissolved CH_4 were recovered and quantified. The amount of CH_4 expected from the added chlorophenol during the 46-day period was determined based on the stoichiometry of the following reaction:

$$C_6H_4OHCl + 4.5\ H_2O \text{ ------> } 3.25\ CH_4 + 2.75\ CO_2 + HCl$$

The stoichiometry was calculated from the Buswell equation (Reference 83) as reported by Shelton and Tiedje (Reference 1), assuming that one H_2 produced from the oxidation of phenol was used for dechlorination rather than methane production, as reported for the dechlorination and degradation of 3-chlorobenzoate (Dolfing and Tiedje, in press).

The fate of ^{14}C-para-chlorophenol was examined in Bioreactor II was examined by determining. The study took place from Day 157 through Day 202 of operation with an average influent substrate of 15.5 mg l^{-1} day^{-1} and average HRT of 7.2 days. The substrate conversion efficiency was 99 percent. Para-chlorophenol was added to 300 mL of media containing 53.4 Ci of [U]-^{14}C-para-chlorophenol and 150 mg/L of total chlorophenol (1:4:1 mixture of ortho-: meta-: para-), and was delivered to the bioreactor over a 72-hour period.

Labeled material was recovered in a series of traps which were connected to the bioreactor. Gaseous products were separated from the effluent by means of the gas release structure (Figure 24). Gaseous products passed through a series of CO_2 traps (vials containing scintillation fluid) before collection of non-CO_2 gases in the inverted cylinder. Samples of the gas collected in the inverted cylinder were periodically placed in 3 mL Venoject vials (Terumo Medical Co.) for later analysis. Radioactivity was measured in the samples stored in Venoject vials by oxidizing the gas to CO_2 in a Harvey Biological Material Oxidizer Model OX200 and collecting the resultant CO_2 in scintillation fluid.

The effluent, containing any dissolved ^{14}C, was collected in a sealed Erlenmeyer flask. The flask was connected to a series of CO_2 traps followed by an additional series of traps (scintillation vials containing 20 mL of: 0.375 gram 2,5-diphenyloxazole, 0.100 gram dimethyl POPOP in 1.0 liters of toluene) to collect non-CO_2 volatiles. Before daily collection of the effluent, tubing to the bioreactor was clamped off, the effluent was acidified with HCl and flushed with nitrogen gas. The effluent was then collected, the

volume was measured and duplicate 1.0 mL samples were counted in scintillation fluid. Radioactivity was measured using a Beckman Model LS8100 Scintillation Counter. Effluent samples were also frozen for later chlorophenol analysis.

4. Trichlorophenols and Pentachlorophenol Studies

Three experiments were run with Bioreactor II to determine the fate of higher chlorinated phenols in the bioreactor. During these experiments, influent loading rates of the monochlorophenol substrates were decreased. Between experiments, the loading rates of the monochorophenols were increased to approximately 20 mg total substrate l^{-1} day^{-1}.

The higher chlorinated phenol substrates were added to the feed media just prior to use from stock solutions. Effluent was monitored for monochlorophenol, the higher chlorinated substrate and any possible chlorinated products.

5. Substrate Loading Rate and Hydraulic Retention Time Studies

The capacity of the bioreactors to degrade chlorophenols at different loading rates and HRTs was examined in Bioreactor III. Influent substrate at a constant HRT was varied by changing the concentration of chlorophenol in the feed media. HRT was varied by changing the volume of feed media pumped into the bioreactor.

6. Location of Biomass

After termination of experiments, the bioreactors were disassembled to measure microbial activites. Bioreactor III was divided into five nonequal portions according to location in the bioreactor. Portions of each section were collected for analysis of protein concentration, methane production, and dechlorinating activity.

Methane production and dechlorinating activity were determined by placing duplicate 25 mL portions of each section into 71 mL serum bottles using strict anaerobic techniques. Meta-chlorophenol was added to each bottle to a final concentration of 48 μg mL^{-1} before the bottle was sealed with a butyl rubber stopper and incubated at 37°C in the dark. Methane production was monitored as previously described. Samples of the aqueous phase were taken periodically and frozen for later determination of dechlorinating activity. The samples were thawed, extracted with 0.5 mL of acetonitrile, filtered through Millipore HVLP filters and assayed by HPLC as previously described.

7. Biomass Morphology

At the termination of the bioreactor experiments, the biomass was observed with a Leitz Ortholux microscope. Tentative identification of microorganisms was made based on the unique morphologies of the restricted enriched community, whose members were compared to batch enrichments, their isolates and known microorganisms.

C. RESULTS AND DISCUSSION

Three anaerobic upflow bioreactors operated for 350, 400, and 190 days, respectively, demonstrating that dechlorinating activity and the anaerobic conditions necessary for this activity can be maintained in the bioreactors for extensive time periods with chlorophenols as the sole carbon and energy source. The feed substrate concentrations varied from 30-150 mg l^{-1} and HRT varied from 2-10 days to accommodate an initial start-up period and changes in the chlorophenol content of the effluent, to determine the range of feasible substrate loading rates into the bioreactors, and to perform various experiments. We were concerned with both washout of the biomass from short HRTs and toxicity of the chlorophenol substrate from high loading rates. The substrate loading rate into the bioreactor was determined by both the substrate feed concentration and the HRT. Thus, results for the bioreactors are described as the influent and effluent substrate loading rate in mg l^{-1}.

In the first two bioreactor experiments, we attempted to determine a range of feasible substrate loading rates and HRTs whereas maintaining a substrate conversion efficiency of greater than 90 percent. Short-term evidence, with Bioreactor I, suggested that a substrate loading rate of 60 mg l^{-1} day^{-1} at a HRT of 2 days was possible (Figure 25). During long-term mineralization studies, (Days 110 through 210), we demonstrated that substrate loading rates of 10 mg l^{-1} day^{-1} at a HRT of 4 days could be maintained at 100 percent substrate conversion efficiency. With Bioreactor II, short-term evidence also suggested that substrate loading rates of 70 mg l^{-1} day^{-1} at a HRT of 2 days and a substrate conversion efficiency of 100 percent was feasible (Figure 26). These loading rates were achieved using a mixture of all three monochlorophenols. The mineralization studies conducted during Days 112-202, demonstrated that loading rates of approximately 18 mg l^{-1} day^{-1} at a HRT of approximately 8 days and a substrate conversion efficiency of approximately 100 percent could be maintained.

To more fully define maximum substrate loading rates at a specific HRT, experiments were performed with Bioreactor III, which utilized only meta-chlorophenol as the sole carbon source (Table 12). Loading rates of 20 mg l^{-1} day^{-1} at HRTs of 2 and 4 days were achieved at substrate conversion efficiencies of greater than 90 percent. However, when the loading rate was increased to 30 mg l^{-1} day^{-1} at both retention times, the substrate conversion efficiency decreased to approximately 60 percent. It appeared that the biomass was adapted to degrade only 20 mg l^{-1} day^{-1} because the decrease in substrate conversion efficiency from 95 to 65 percent was equal to the difference between 20 and 30 mg l^{-1} day^{-1}. Toxicity of the substrate at the higher loading rate did not appear to affect the biomass because it retained the ability to degrade the lower substrate loading rate of 20 mg l^{-1} day^{-1}. In future experiments it may be possible to achieve higher substrate loading rates in bioreactors by either allowing a sufficient period of time for the biomass to adapt to a higher substrate loading rate or by some other means of increasing the amount of active biomass in the bioreactor.

TABLE 12. CHLOROPHENOL REMOVAL EFFICIENCY AT DIFFERENT SUBSTRATE LOADING RATES AND HYDRAULIC RETENTION TIMES (HRT) IN BIOREACTOR III.

Expt #	Substrate Loading Rate (mg l^{-1} d^{-1})	Effluent (mg l^{-1} d^{-1})	HRT (days)	Removal Efficiency (percent)
1	21.1	1.01	1.9	95.2
2	27.1	9.28	2.3	65.8
3	19.4	1.25	4.1	93.6
4	27.1	6.46	4.6	76.2

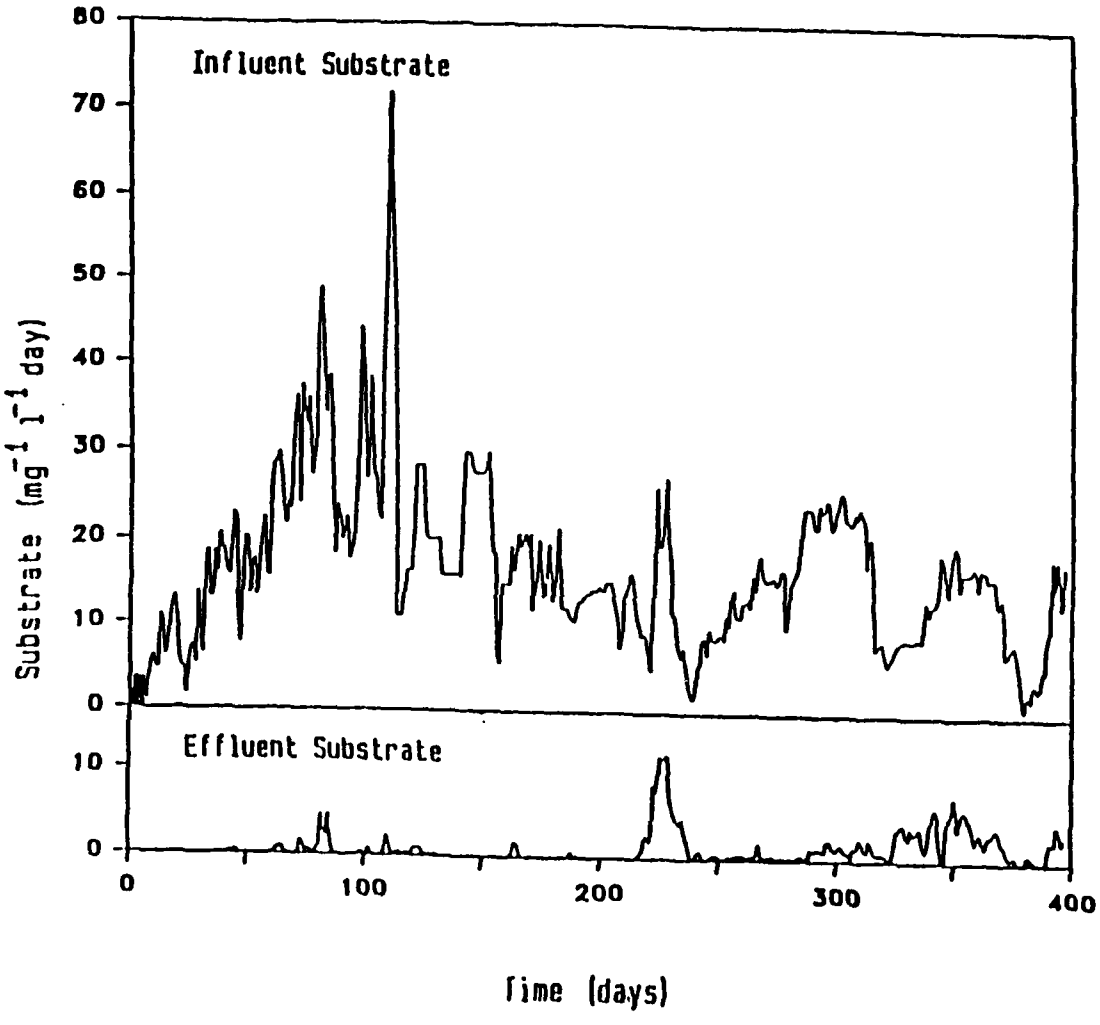

Figure 25. Bioreactor I Influent and Effluent Substrate Loading Rate. The Substrate was meta-Chlorophenol.

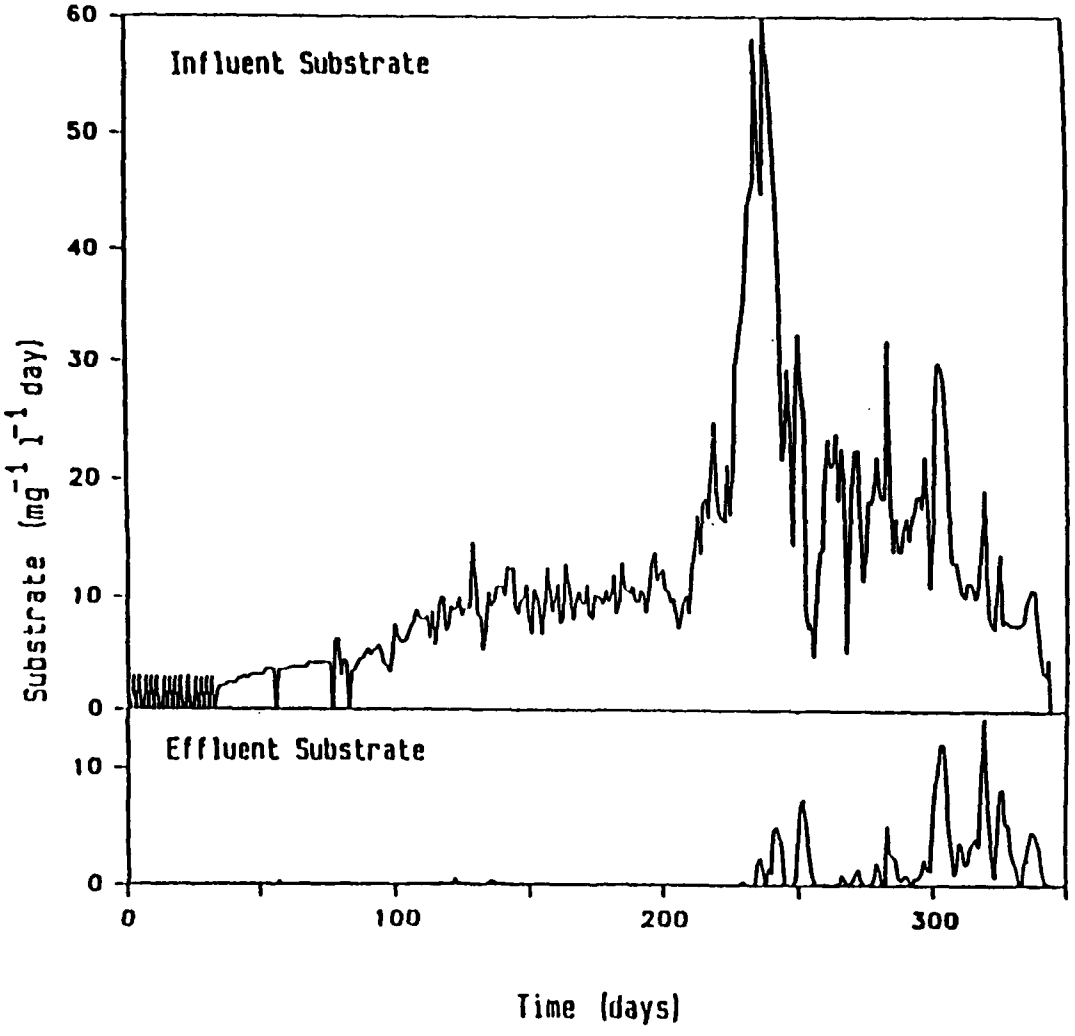

Figure 26. Bioreactor II Influent and Effluent Substrate Loading Rate. The Substrate Was a 1:4:1 Mixture of ortho-:meta-: para-Chlorophenol.

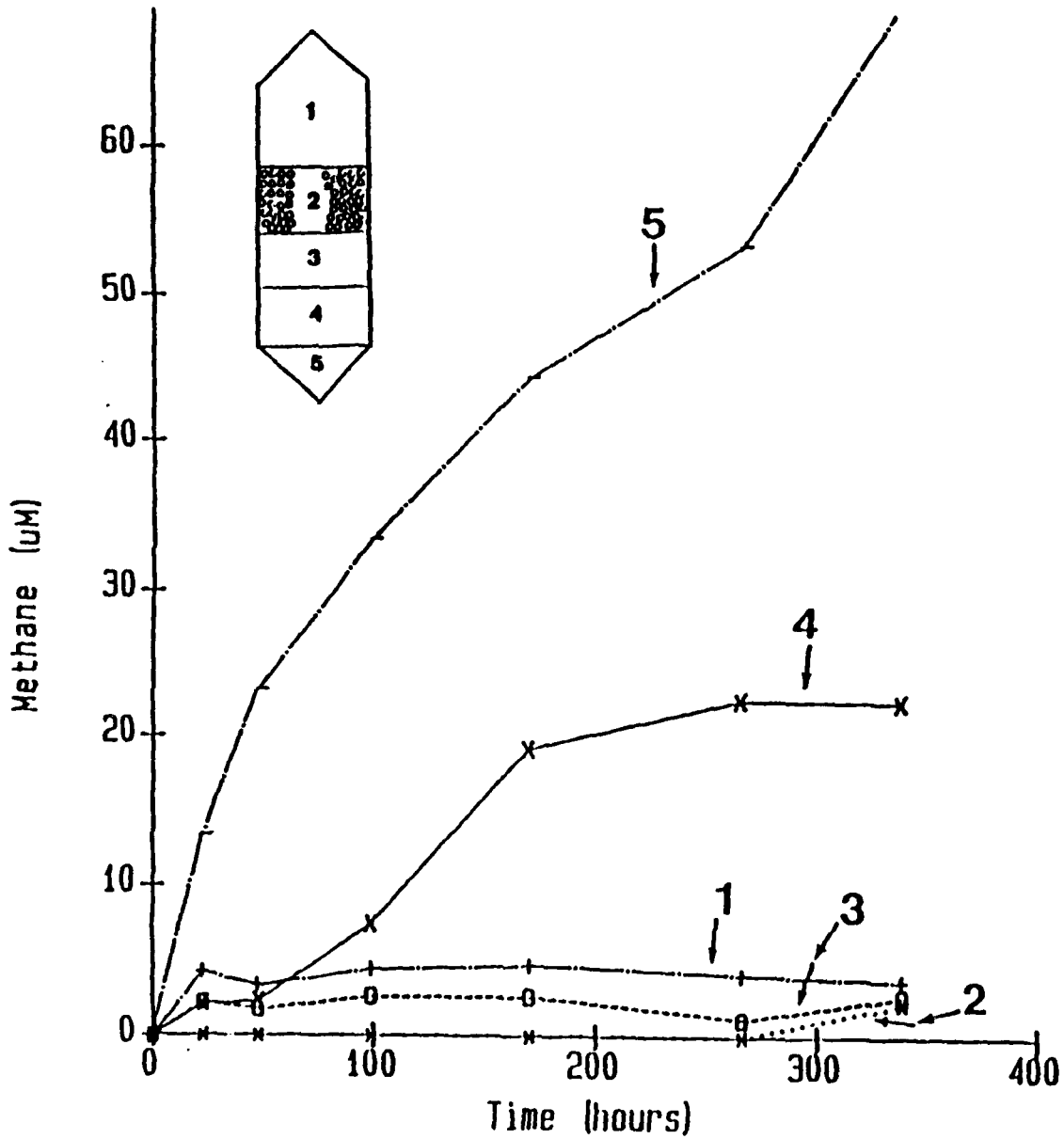

Figure 27. Methanogenic Activity per Column Section in Bioreactor II after 190 Days of Operation. Numbers refer to positions in the column.

The chlorophenols were mineralized to CH_4 and CO_2 in the bioreactors. In Bioreactor I, approximately 39 percent of added chlorophenol substrate could be accounted for as gaseous end products based on the recovery of methane (Table 13). Similarily, in Bioreactor II, 44 percent of added ^{14}C-para-chlorophenol could be accounted for as gaseous ^{14}C products based on the recovery of $^{14}CO_2$ (Table 14). Mineralization of the chlorophenol to CH_4 and CO_2 demonstrated that chlorophenol disappearance was due to biodegradation and was additional evidence that strict anaerobic conditions were maintained in the bioreactors.

Dehalogenating activity was maintained on a 1:4:1 mixture of ortho-; meta-; para-chlorophenol for 400 days in Bioreactor II (Figure 26) suggesting that anaerobic upflow bioreactors may be capable of treating more complex waste streams containing several chlorinated phenols because dechlorination occurred at all positions on the aromatic ring. Batch culture experiments with acclimated anaerobic sewage sludge have demonstrated the ability to dechlorinate higher chlorinated phenols such as the dichlorophenols and to dechlorinate and partially mineralize pentachlorophenol (References 19, 33, 84). Thus, the potential for dechlorinating higher chlorinated phenols exists.

Results from the addition of higher chlorinated phenols to Bioreactor II are summarized in Table 15. There was minimal or no biodegradation of either 2,4,6-trichlorophenol or pentachlorophanol. Approximately 65-75 percent of added substrate was detected in the effluent. In contrast, the 3,4,5-trichlorophenol was degraded; only 28 percent of added 3,4,5-trichlorophenol was recovered in the effluent. The lack of dechlorination of 2,4,6-trichlorophenol and pentachlorophenol in the bioreactors may be the result of improper experimental conditions such as the loading rate, the HRT or toxicity of the substrate. The substrate conversion efficiency for the monochlorophenols decreased upon the addition of each of the higher chlorinated phenols, suggesting that the trichlorophenols and pentachlorophenol may have been toxic to the dechlorinating activity (Figure 26).

The majority of the methanogenic and dechlorinating activity in Bioreactor III was located at the bottom of the bioreactor near the screen designed for the accumulation of biomass in the form of a sludge blanket (Figures 27 and 28). Similar results were obtained with Bioreactor I and when protein measurements were used as an indication of the biomass (data not shown). There was little or no activity associated with the glass beads indicating that when HRTs of greater than two days are used the glass beads can be removed without loss of activity.

The bioreactors enriched for a specific population of bacteria. Although other microorganisms were present, three microbes dominated the bioreactor contents. These appeared to be: (1) a putative phenol-degrader; isolated in pure-culture with a hydrogen-consumer from phenol-degrading enrichments (Reference 85), (2) a hydrogen-utilizing and acetoclastic methanogen, similar in morphology to Methanosarcina sp. and (3) a acetoclastic methanogen, recognized by its Methanothrix-like morphology.

Anaerobic upflow bioreactors can be used as a tool in studying unique microorganisms and their activities. Bioreactors are an especially important

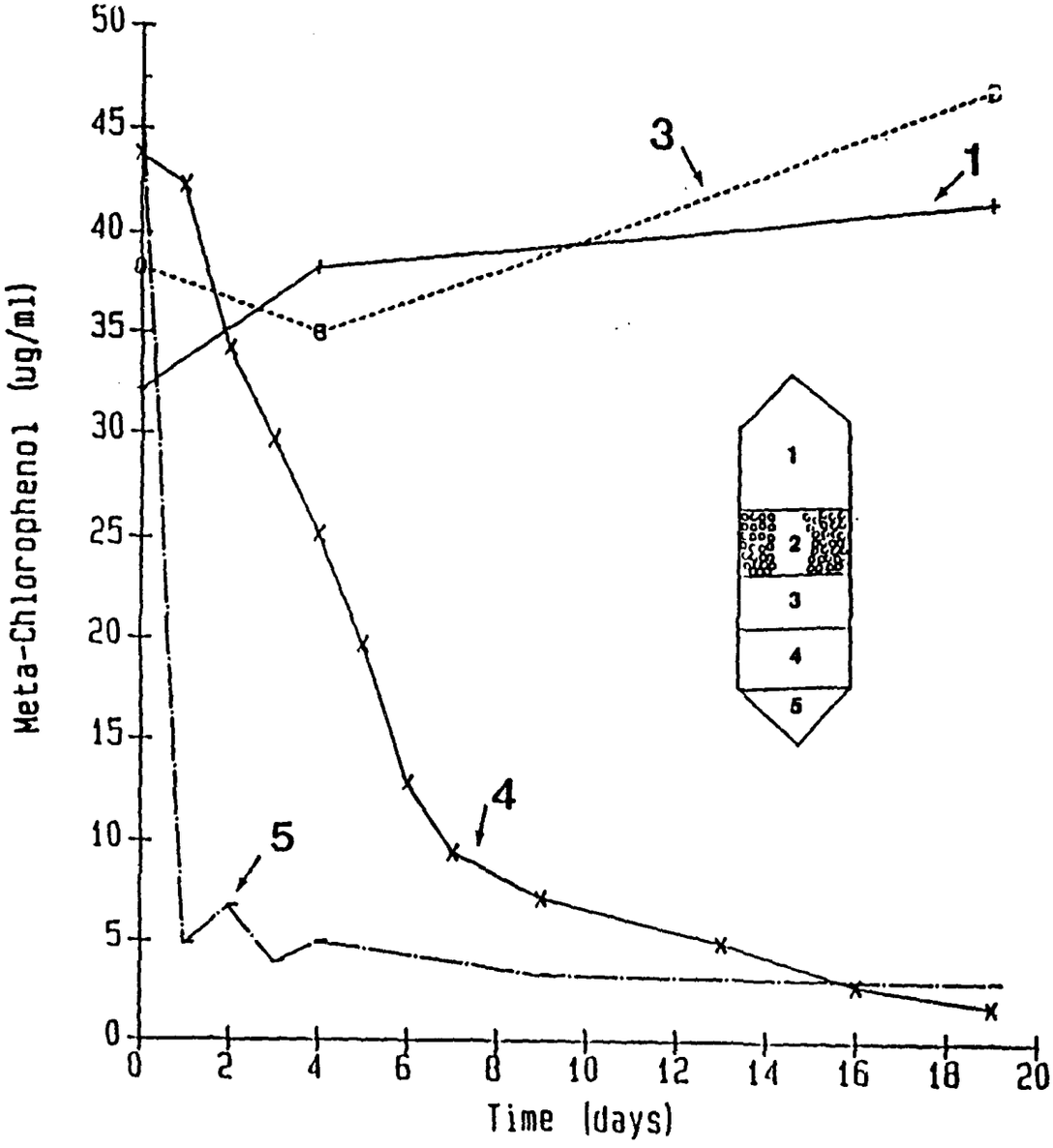

Figure 28. meta-Chlorophenol Activity per Column Section in Bioreactor II after 190 Days of Operation.

TABLE 13. MINERALIZATION OF M-CHLOROPHENOL TO GASEOUS END PRODUCTS IN BIOREACTOR I.

	Gaseous End Product Yield (percent of theoretical)
Methane	22 percent
Carbon Dioxide*	17 percent
Total	39 percent

*CO_2 was calculated from the methane.

TABLE 14. FATE OF ^{14}C-PARA-CHLOROPHENOL IN BIOREACTOR II.

Fraction	Percent of Added Label
Effluent (p-chlorophenol)	6.1 percent
CO_2	19.4 percent
Non-CO_2 Volatile	4.1 percent
Calculated CH_4	24.9 percent
Total Gaseous	44.3 percent

TABLE 15. FATE OF HIGHER CHLORINATED PHENOLS IN BIOREACTOR II.

Substrate	Days of Operation (days)	Average Influent Load (mg l^{-1} d^{-1})	HRT* (days)	Total Substrate (mg)	Substrate Conversion Efficiency (percent)
2,4,6-Trichlorophenol	213-222	6.28	3.6	56.5	23.2
3,4,5-Trichlorophenol	315-329	2.04	2.2	30.7	71.52
Pentachlorophenol	371-389	1.61	2-5	30.6	35.2

*Hydraulic retention time

tool when the microbial activity of interest is the result of interactions between members of microbial communities. When the microbes responsible for a unique activity are difficult to identify in a microbial community and isolate by conventional methods, such as the ones studied here, bioreactors may be the only feasible method of studying these unique microbial activities.

Anaerobic upflow bioreactors can be an alternative to conventional enrichment culture techniques where portions of the enrichments are transferred periodically into fresh media. The undesirable microbes and metabolic end-products are eliminated and the microbes of interest are selected. Conventional enrichment culture techniques may be lengthy due to slow growth of the organisms after perturbation of tha enrichment. Enrichment of the desirable microbial community can be achieved faster in anaerobic upflow bioreactors for two reasons. First, the majority of the microbial biomass remains in the bioreactor, usually in the form of a sludge blanket or attached to a solid support material. Second, enrichment is continual; substrate is continually added to the bioreactor whereas end-products are continually removed. An upflow bioreactor also provides a feasible method of enrichment when the substrate of interest is toxic to the microbes, as is the case with chlorophenols because low concentrations of the substrate can be continually applied to the bioreactor.

SECTION VII

REDUCTIVE DECHLORINATION OF 4-CHLORORESORCINOL BY ANAEROBIC MICROORGANISMS

A. RATIONALE AND APPROACH

One approach to finding additional dechlorinating capacity is to select for dechlorinating activity in new classes of chemicals that are reasoned to yield promising results. We selected chlororesorcinol as the substrate because the resorcinol product directly yields enough energy to support of a microorganism. Thus, it might be much easier to obtain dechlorinating activity and to isolate the responsible organism.

B. MATERIALS AND METHODS

Fresh anaerobic sewage sludge was obtained from a primary digestor (Jackson MI) in 4-L aspirator bottles. After transport to the laboratory, the headspace was filled, purged with deoxygenated N_2 gas. The bottles were stoppered and stored for 48 hours at room temperature to reduce excess soluble carbon. All test chemicals used in this study were obtained from Aldrich Chemical Co., Inc (Milwaukee WI) and were used without further purification.

1. Fresh Sludge

Degradation of 4-chlororesorcinal (4-Cl-Res) in fresh sludge (48 hours after collection) was studied in a serum bottle incubation system. All incubations were carried out in 160 mL capacity (described as 125 mL Wheaton serum bottles, American Scientific Products) serum bottles which were sparged with deoxygenated N_2 gas using a modified Hungate gassing procedure (Reference 72). One-hundred millliliters of fresh sludge was dispensed into each serum bottle, whereas continuously sparging with the N_2 gas. Test compounds were added from aqueous stock solutions to achieve the desired concentrations. The serum bottles were then sealed with 1 cm thick black butyl rubber stoppers and aluminium caps. All bottles were incubated static in the dark at 37°C. Two-millliliter samples were withdrawn by syringe at various time intervals and stored frozen until analyzed.

2. Development of Enrichment Culture

One liter of fresh sludge contained in an aspirator bottle was initially fed 50 mg (50 ppm) of 4-Cl-Res. The concentration of the added 4-Cl-Res was monitored on a weekly basis and when the compound was completely degraded to below the detection limit (<2 ppm), the bottle was refed to give a final total concentration of 50 ppm. This process of feeding and refeeding was continued for a period of at least 4 to 5 months at which time the rate of degradation had increased appreciably (more than 90 percent of the added chemical was removed within 2 weeks). At this time the sludge was referred to as an acclimated sludge.

The 4-chlororesorcinal degrading microbes were enriched by transferring initially 200 mL of the above acclimated sludge to 800 mL of prereduced enrichment mineral medium (PREM-medium) containing 50 μg mL 1 of 4-Cl-Res as the only exogenous aromatic carbon source. 4-Chlororesorcinol was added to the PREM-medium several times (4-6 times) once the previously added substrate (4-Cl-Res) was degraded completely. Fifty milliliters of this batch enrichment was then transferred to 1 liter (5 percent transfer) of fresh PREM-medium containing 4-Cl-Res (50 $\mu g\ mL^{-1}$), and again refed 4-5 times as the 4-Cl-Res was degraded. This process of feeding and refeeding of substrate and then making a 5 percent transfer of the mixed culture to fresh medium was performed at least six times. At this time the enrichments were calculated to contain only < 0.0001 percent of the original acclimated sludge. Most of our degradation studies were done with this enrichment which was approximately 2 years old.

3. Medium and Cultivation Conditions

The composition of PREM-medium used in this study was the same as that described previously (Reference 86) except that in the present medium 4-Cl-Res served as the only added aromatic carbon-energy source, and the medium contained 0.025 gram yeast-extract per 100 mL to support initial growth of the culture.

In each experiment, 25 mL portions of the stock enrichment culture were anaerobically transferred to 70 mL serum bottles that had been flushed for 10 minutes with deoxygenated N_2 gas. The test chemicals (4-Cl-Res or resorcinol) were added from aqueous stock solutions (stored anaerobically) to achieve the desired concentration. All incubations were at 37°C without shaking in the dark.

4. Effect of Nutrients on the Dechlorination of 4-Chlororesorcinol

The rate of dechlorination of 4-chlororesorcinol was studied in the presence of 0.1 percent yeast extract (YE), 0.1 percent trypticase (TRP), 5 percent rumen fluid (RF), 5 percent sludge supernatant (SS), 0.1 percent glucose (Glu) or 0.002 percent resorcinol.

Aqueous stock solutions of YE, TRP, RF, and SS were sterilized by autoclaving Glu and Resorcinol solutions were filter sterilized (0.45 μ, Millipore).

5. Effects of Yeast Extract and Substrate Concentrations

Several serum bottles containing 50 mL of defined medium (PREM-medium devoid of yeast extract) were supplemented with various amounts of yeast-extract (0 to 0.3 g/100 mL) and inoculated with 1 mL of aged enrichment. Samples (1 mL) were withdrawn periodically and analyzed for 4-Cl-Res depletion and resorcinol formation.

The effect of 4-Cl-Res concentration on dechlorination was studied in serum bottles containing 50 mL of enriched medium (PREM-medium containing 0.1 gram yeast extract/100 mL) which were amended with various amounts of 4-Cl-Res. The substrate chemical was added from an aqueous stock solution. The serum bottles were sampled at the end of 2, 4, and 8 days and analyzed for disappearance of 4-Cl-Res and appearance of products.

6. Determination of Lag Time and Rate of Dechlorination

The lag period was considered to be as the time until measurable substrate depletion was noted. In addition it was confirmed by the appearance of an expected dechlorinated product at quantities greater than 3-5 percent of the added substrate.

The rates of dechlorination were calculated as the time required to dechlorinate > 95 percent of the substrate present at the end of lag period and expressed as μ·moles of substrate disappeared per mg of protein (biomass) per day.

7. Analytical Techniques

The 2 mL sludge sample or 1 mL enrichment samples were combined with 1 mL of acetonitrile and mixed for 30 seconds with a Vortex mixer. The samples were then centrifuged for 10 minutes at 12,000 x gram and filtered through 0.45 m Millipore filters (Type HVLP). Substrate (4-Cl-Res) depletion and product (Res) formation was monitored by reverse phase high-performance liquid chromatography (HPLC). The HPLC system (Waters Associates, Inc., Milfort MA) was equipped with a model 6000A pump and a model 441 absorbance detector. All compounds were detected by their absorbance at 280 nm. The samples were injected via a Rheodyne 7125 sample injector (Rheodyne, Cotati, California) fitted with a 20 μL loop. The analytical column was a Waters Radial-PAK, C-18 Cartridge held in an RCM-100 radial compression module. Peak areas were measured with a Hewlett-Packard 3390A integrator. An external standard procedure was used for peak identification and quantitation. The mobile phase consisted of a 2:1 mixture of 2 percent aqueous acetic acid and methanol. A flow rate of 2 mL/min gave retention times of 4.54 minutes and 2.35 minutes, for 4-Cl-Res and Res, respectively.

C. RESULTS AND DISCUSSION

The anaerobic biodegradation of 4-Cl-Res (380 μM) in fresh sludge required approximately 4 weeks and occurred without a lag period (Figure 29). 4-Chlororesorcinol was completely dechlorinated and resorcinol was recovered in almost stoichiometric amounts. A second addition of 4-Cl-Res prior to the removal of the previously formed product (Res) resulted in complete dechlorination in 2 weeks, i.e., at a rate 2X faster than the initial feeding. The reductive dechlorination of 4-Cl-Res was again demonstrated by the stoichiometric accumulation of Res (700 μM) from the two feedings (Bottle A, Figure 29). The improved rate of dechlorination after refeeding suggested an acclimation process. The dechlorinated product resorcinol underwent only a partial degradation (about 29 percent) even when incubated for an additional 10 weeks after the disappearance of 4-Cl-Res.

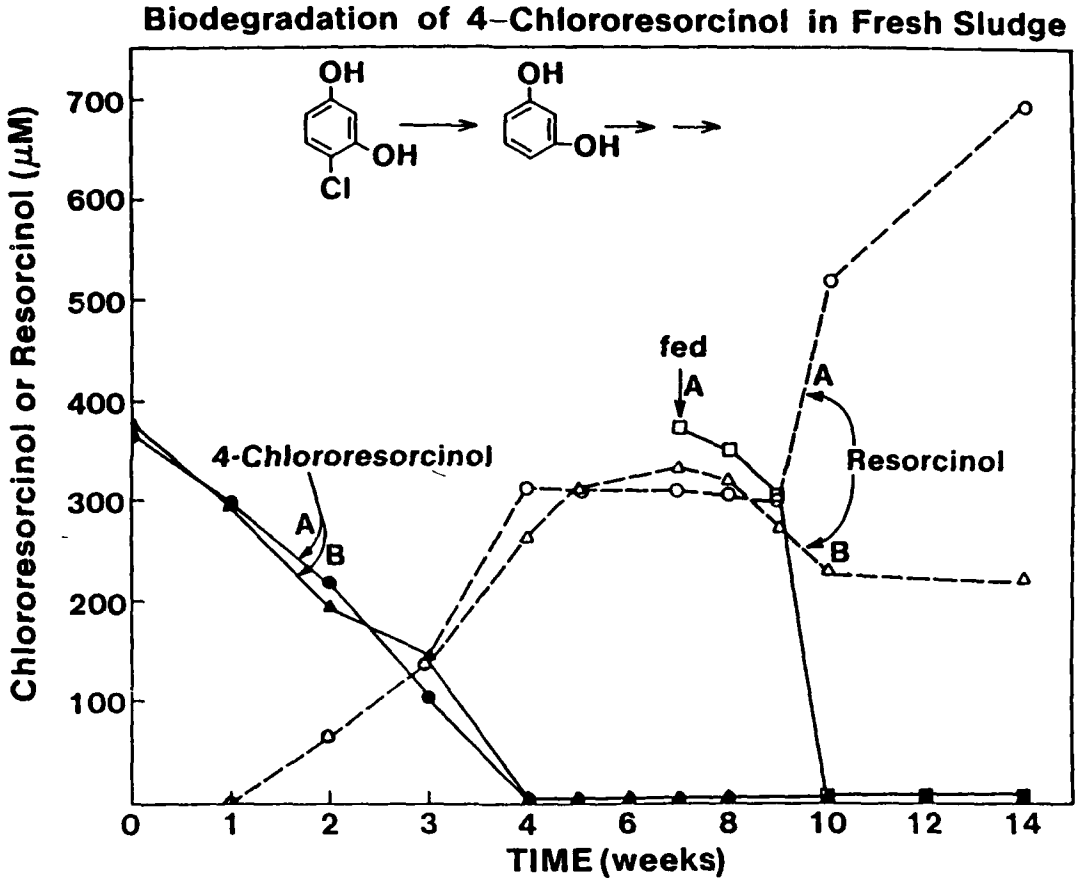

Figure 29. Biodegradation of 4-Chlororesorcinol in Fresh Sludge.

Figure 30 represents the pattern of 4-chlororesorcinol degradation by the enrichment. The substrate was completely dechlorinated stoichiometrically to resorcinol within a 4-week period. The lag times prior to measurable substrate depletion and product formation ranged between 20 to 25 days and then more than 95 percent of the added substrate was dechlorinated within the next 4 to 6 days. The rate of dechlorination during this period was estimated to be around 0.3 μmole/mg/day. However, feeding the enrichments soon after depletion of the previously fed substrate resulted in immediate dechlorination of the substrate. In the present study, refed 240 μm 4-chlororesorcinol was completely dechlorinated within 5-6 days at a rate of 0.27 μmole/mg/day. Interestingly, resorcinol was not accumulated to stoichiometric amounts after the second feeding suggesting a high activity of ring degraders. Our studies also indicated that if the enrichment was fed after a whereas (@ 1 week) after dechlorination of the previously fed substrate resulted in a long lag for dechlorination (at 20 days).

Microscopic observations of the enrichment showed fewer types of morphologically different organisms characterized by many short and long rods in addition to spore formers. Organisms similar to Methanosarcina and Methanothrix were the major methanogenic species.

1. Effect of Nutrient Carbon

These studies show that the addition of 0.1 percent YE, 0.1 percent TRP or 5 percent RF to the enrichment resulted in complete dechlorination within 2 to 3 days of incubation. Similar enrichments amended with 0.1 percent Glu, 5 percent SS or .002 percent resorcinol (aromatic carbon) stimulated dechlorination activity but at a slower rate. Complete dechlorination of 4-Cl-Res occurred by day 8, 12, and 16 in the presence of Glu, SS, and Res, respectively. The observed lag periods were approximately 3 days for Glu and SS and 8 days for resorcinol. Addition of acetate (50 mM) neither stimulated nor inhibited the dechlorination of 4-Cl-Res under the experimental conditions.

A closer examination of the effect of YE and TRP on dechlorinating activity is shown in Figure 31. These results show that 280-295 μm 4-chlororesorcinol was completely biotransformed to resorcinol within 48 hours in the presence of 0.1 percent YE or 0.1 percent TRP. The lag periods were approximately 24 and 36 hours for YE and TRP, respectively.

Although the lag period in the presence TRP was longer, the rate of dechlorination was twofold (2.58 μmole/mg/day) higher than the rates in the presence of YE (1.13 μ·mole/mg/day). The biomass content in the presence of YE and TRP was the same. In both cases stoichiometric amounts of product were formed. The product thus, formed persisted for longer time (@ 1 week) suggested that the addition of nutrients such as YE and TRP did not help stimulate the ring-degrading activity.

Concentrations of yeast extract ranging from 0.0 percent to 0.3 percent were tested for their effect on the dechlorination of 4-Cl-Res. Our results (not shown) clearly showed a concentration dependent stimulation of

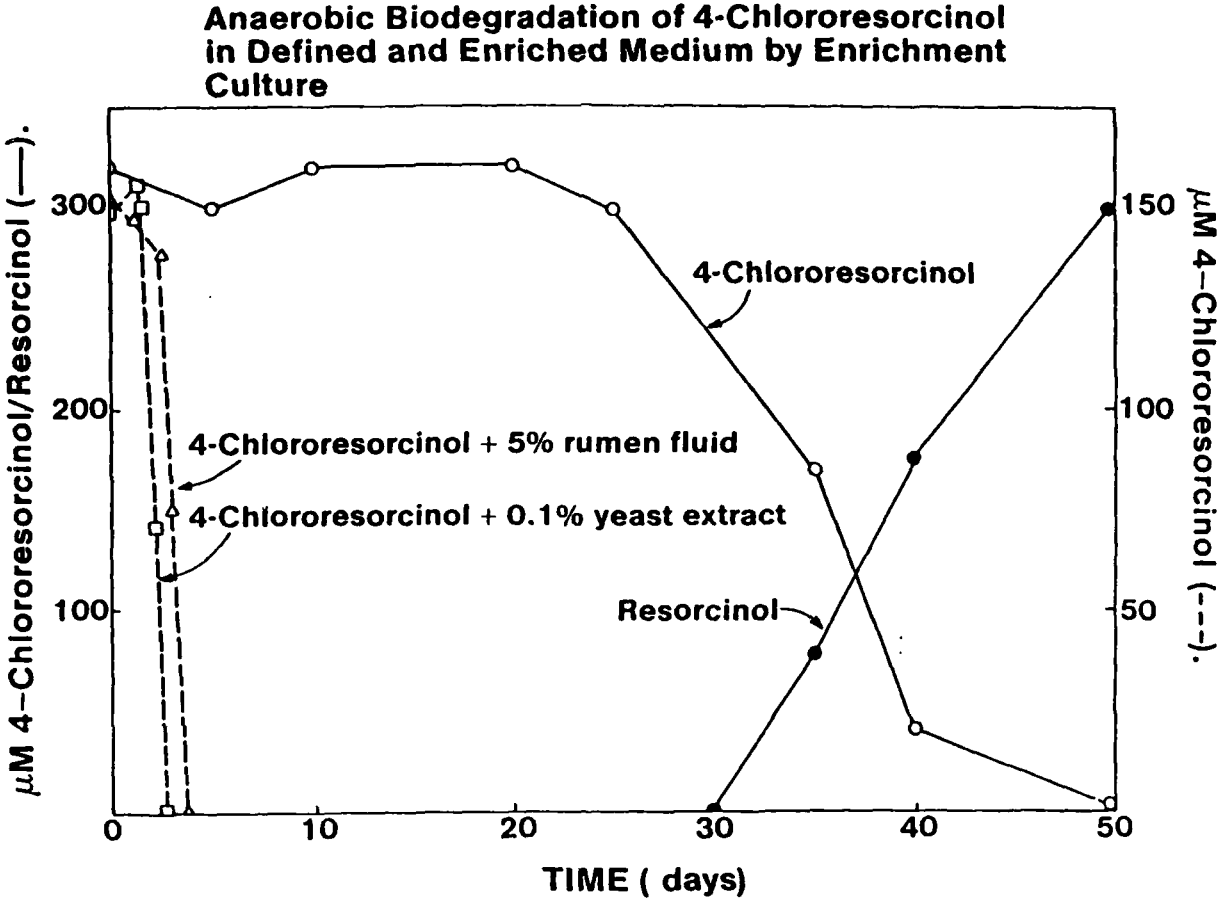

Figure 30. Anaerobic Biodegradation of 4-Chlororesorcinol in Defined and Enriched Medium by Enrichment Culture.

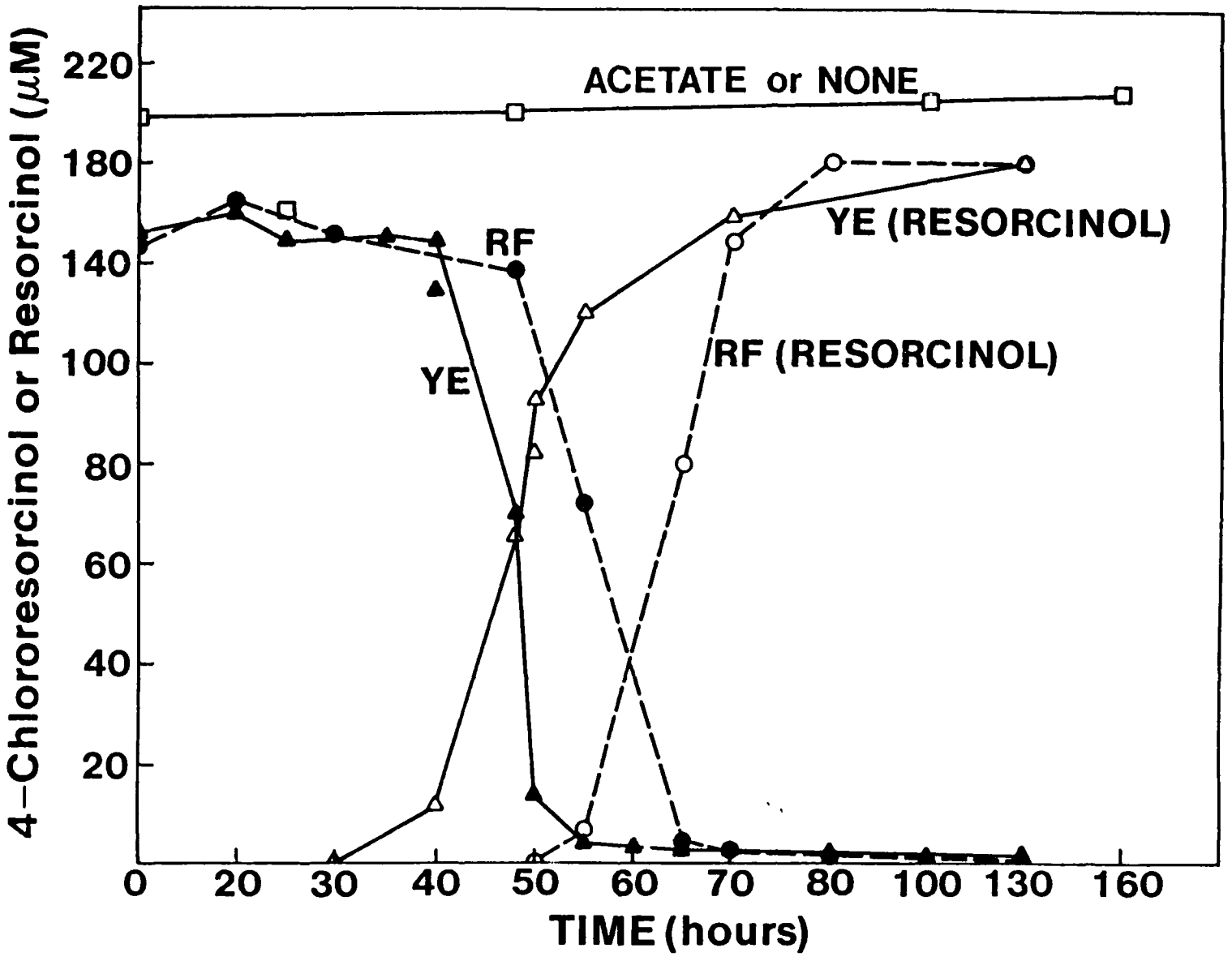

Figure 31. Anaerobic biodegradation of 4-Cl-resorcinol by enrichment culture in the presence of yeast extract and rumen fluid.

dechlorinating activity. The minimum amount of the yeast extract required to achieve the maximum percent dechlorination was 0.1 percent. No further improvement in dechlorinating activity was observed above 0.1 percent. Dechlorination of 4-Cl-Res at all tested yeast extract concentrations gave stoichiometric conversions to Res.

4-Cl-Res was completely dechlorinated within 2 days with a lag period of 1 day. The rates of dechlorination increased with increasing concentration of substrate and reached maximum of 4.5 to 5.0 $\mu moles \cdot mg^{-1}$ day^{-1} at initial 4-Cl-Res concentration of 200 M. These results suggest that the rate of conversion is substrate limited below 200 μM 4-Cl-Res. Maximum initial rates of dechlorination was observed at substrate concentration between 200 to 900 μM. However, after the initial maximum rate, the rate of dechlorination decreased slowly.

The extent of dechlorination was 0, 36, and 73 percent at 700, 500, and 300 μM 4-Cl-Res, respectively at the end of 2 days. Complete transformation occurred at concentrations up to 500 μM when the incubation time was extended to the 4th day. Similarly, more than 8 days was needed for the complete dechlorination of 4-Cl-Res Al-concentrations between 700 to 900 μM after a lag time of 2 days. Addition of higher levels of substrate. i.e. 900 to 1500 μM (results not shown) had a longer lag time (> 4 days) and lower rates of conversion. Above 1500 μM no dechlorination occurred even after 10 days of incubation indicating a toxic effect at higher concentrations. Lag periods were 1, 2, > 4 and > 10 days for initial 4-Cl-Res concentrations of < 200, > 200, > 700, and > 1500 μm, respectively.

Enrichment cultures were intially amended with 4-Cl-Res, resorcinol or 4-Cl-Res plus resorcinol and incubated for various lengths of time to study the effeet of resorcinol on dechlorination of 4-Cl-Res. All chemicals were added at equal molar concentrations. The dechlorination of 4-Cl-Res proceeded with a shorter lag time (8 days) and was completed within 20 days. There were an initial decrease in resorcinol concentration and then a transitory aocumulation of resorcinol. The amount of accumulated resorcinol at the end of 20 days (146 percent) was almost double the amount detected on the 8th day (74 percent). These results show that the accumulated resorcinol was the dechlorinated product of 4-Cl-Res, and during this active dechlorination phase, degradation of resorcinol had either not occurred or was degraded at very low rates. Once the dechlorination was completed, the degradation took place. Resorcinol was biodegraded within 7 to 8 days, with little or no lag time, when it was added alone. On the contrary, dechlorination of 4-Cl-Res began after approximately 20 days, and was complete within the next 5 to 6 days.

These observations indicate that the enrichment was composed of both active dechlorinator(s) and ring degrader(s). In addition, the dechlorination step is an essential and a rate limiting step for ring cleaving processes.

Dehalogenation is an important reaction that has now been shown to occur in most anaerobic environments with an increasingly diverse family of substrates (Reference 87). In the present study we have demonstrated the

anaerobic dechlorination of 4-chlororesorcinol in fresh anaerobic digestor sludge and in highly enriched microbial communities. Dechlorination of 4-Cl-Res was the primary step in the degradative sequence, as observed previously for chlorinated benzoates and for chlorinated phenols. Thus, these observations provide additional evidence for the general scheme of ring simplification prior to ring fission under anaerobic conditions.

Dechlorination of 4-Cl-Res in fresh sludge occurred without a lag at a rate of approximately 100 μM per week. This was almost identical to the dechlorination of 2-chlorophenol by this same sludge (Reference 33) with respect to both the lack of a lag period and to rate of disappearance. The dechlorination of 4-Cl-Res was not inhibited by the accumulation of primary degradation product (Res) suggesting that the dechlorinating organism were different from those involved in ring metabolism. This is further supported by the fact that the rate of dechlorination increased after the second addition of 4-Cl-Res whereas Res had accumulated almost stoichiometrically.

Highly enriched consortia were obtained with 4-Cl-Res as the sole C and energy source. Dechlorination usually began after a lag of approximately 2-3 weeks, unless fed otherwise immediately. The lag times for the dechlorination were dependent on the time intervals between feedings. Delayed refeeding of the culture resulted in late onset of dechlorination of the substrate. One of the reasons for the accumulation of chlororesorcinol the first cycle of dechlorination of chlororesorcinol could be the nonavailability of carbon to support the growth of ring cleavers.

A marked stimulation in dechlorinating activity was observed after addition of nutrient carbon sources to the enrichment. Addition of 0.1 percent YE, 0.1 percent TRP, or 5 percent RF increased the rate of dechlorination by at least 4 to 8 fold and reduced the lag period from 3 weeks to less than 2 days. Our studies, carried out with various concentrations of YE suggested a concentration dependent dechlorination up to 0.1 percent added YE. Addition of higher levels of YE did not further stimulate nor shorten the lag time. Not all carbon sources caused this enhancement because Glu, SS, RES, or acetate had little or no stimulatory effect. The dechlorination activity of the enrichment amended with different nutrients was 2.58, 1.13, 0.42, 0.19, and 0.07 $\mu mole \cdot mg^{-1}\ day^{-1}$ for TRP, YE, RF, Glu, SS, and Res, respectively. Addition of acetate (50 mM) neither stimulated nor inhibited the dechlorination activity under the experimental conditions. This stimulation is very important because the essential practical goal is to enhance dechlorination rates. This is the first example where a greatly enhanced rate has been demonstrated.

The explanation for this stimulation is not clear. It could be due to addition of growth factors for the dechlorinating population or activity. Or, it could be simply a need for additional energy or reductant by the dechlorinating population. Both yeast extract and rumen fluid are well known sources of growth factors for fastidious organism and the factor both simulates dechlorination providing some suport for the first explanation, but does not directly rule out latter explanations. The elucidation of this mechanism is important to achieving reliable schemes to stimulate dechlorination.

Our studies relating to the effect of product (Res) on transformation of 4-Cl-Res showed an enhanced rate of dechlorination. It is, therefore, reasonable to assume that the ring degraders could be cross-feeding the dechlorinating organism(s) with carbon and energy. The dechlorination of 4-Cl-Res is apparently fortuitous, and results in the stoichiometric accumulation of the corresponding dehalogenated aromatic compound. resorcinol. According to Tscheck and Shink (Reference 88), the resorcinol could be fermented to acetate and butyrate in an exergonic reaction. ($G^{o'}$ = -102.3 k.J. mol^{-1}). As mentioned, addition of acetate neither stimulated nor inhibited the 4-Cl-Res transformations. Therefore, the fermentation product of resorcinol other than acetate could be serving as carbon and energy source for the dechlorinator (Reference 1).

Recently isolated 3-chlorobenzoate dechlorinating bacterium (DCB-1) could be a best example of dechlorinator. One interesting feature is that the DCB-1 organism seems to exist only by scavenging products from other members in the food chain, most probably from benzoate degraders (Reference 1). Its dechlorination is also stimulated by rumen fluid. Perhaps this scavenging feature is a common characteristics of dechlorination; if so organic-rich environments might inhibit dechlorination.

It is apparent from these studies that addition of organic nutrients such as yeast extract or rumen fluid: (a) results in enhanced rates of transformation; (b) long lag times are eliminated; (c) active stable microbial community could be maintained for longer periods of time.

SECTION VIII

REDUCTIVE DECHLORINATION OF HEXACHLOROBENZENE IN ANAEROBIC DIGESTOR SLUDGE

A. RATIONALE AND APPROACH

Our previous work had been limited to haloaromatic substrates with polar functional groups (benzoates, phenols, resorcinols) which are different in this chemical character from TCDD. Therefore, it was important to use a surrogate that was nonpolar and highly chlorinated to see if dechlorination occurred. Hexachlorobenzene was used as this substrate and its dechlorination in sludge.

B. MATERIALS AND METHODS

Fresh sludge obtained from primary digestor (Jackson MI) was employed for the anaerobic biodegradation of hexachlorobenzene (Aldrich Chemical Co., Milwaukee WI). All incubations were carried out under stringent anaerobic conditions using 1-L aspirator bottles (digestors) with 700 mL working volume of the sludge. Hexachlorobenzene (HCB) was directly added to each digestor at a concentration of 50 ppm. Control digestors were set up by adding HCB (50 ppm) to the sterile sludge (autoclaved and/or 2 percent formaldehyde treated sludge). All incubations were done at 37°C, in the dark.

1. Extraction and Analysis

Sludge samples (20 mL) were withdrawn anaerobically after 0, 1, 2, 3, 4, 6, 8, 10, 12, 14, and 16 weeks. The sample was manually mixed with 10 mL toluene manually for 3 minutes and centrifuged at 1200 x gram for 15 minutes at 4°C. The supernatant (organic layer) was carefully drained off into a screw-capped container. The residue was twice again extracted with 10 mL of toluene as before. The organic layers were pooled and appropriately diluted in toluene before injection into a gas chromatograph.

The HCB and dechlorinated products were identified and quantified by injecting 2 µL of appropriately diluted samples into a Varian model 3700 gas chromatograph equipped with a ^{63}Ni electron capture detector (ECD). The compounds were separated on a packed column containing 1 percent SP-1000 on 100/120 Supelcoport. The metal column was 2.0 m x 1/4-inch OD x 2 mm ID. The column temperature was held at 100°C for 3 minutes then to raised 200°C at 10°C/min. The injector port temperature was 22°C, and detector temperature was 23°C. N^2 was used as a carrier gas at flow rate of 30 mL/min. Quantitation was by comparison with authentic standards.

C. RESULTS AND DISCUSSION

Studies on the reductive dechlorination of HCB were conducted using sludge (Jackson, Michigan) that had previously shown the ability to dehalogenate chlorinated phenols (Figure 32). The HCB was dechlorinated, as shown by the accumulation of 1,3,5-trichlorobenzene (TCB). Approximately 30 percent of

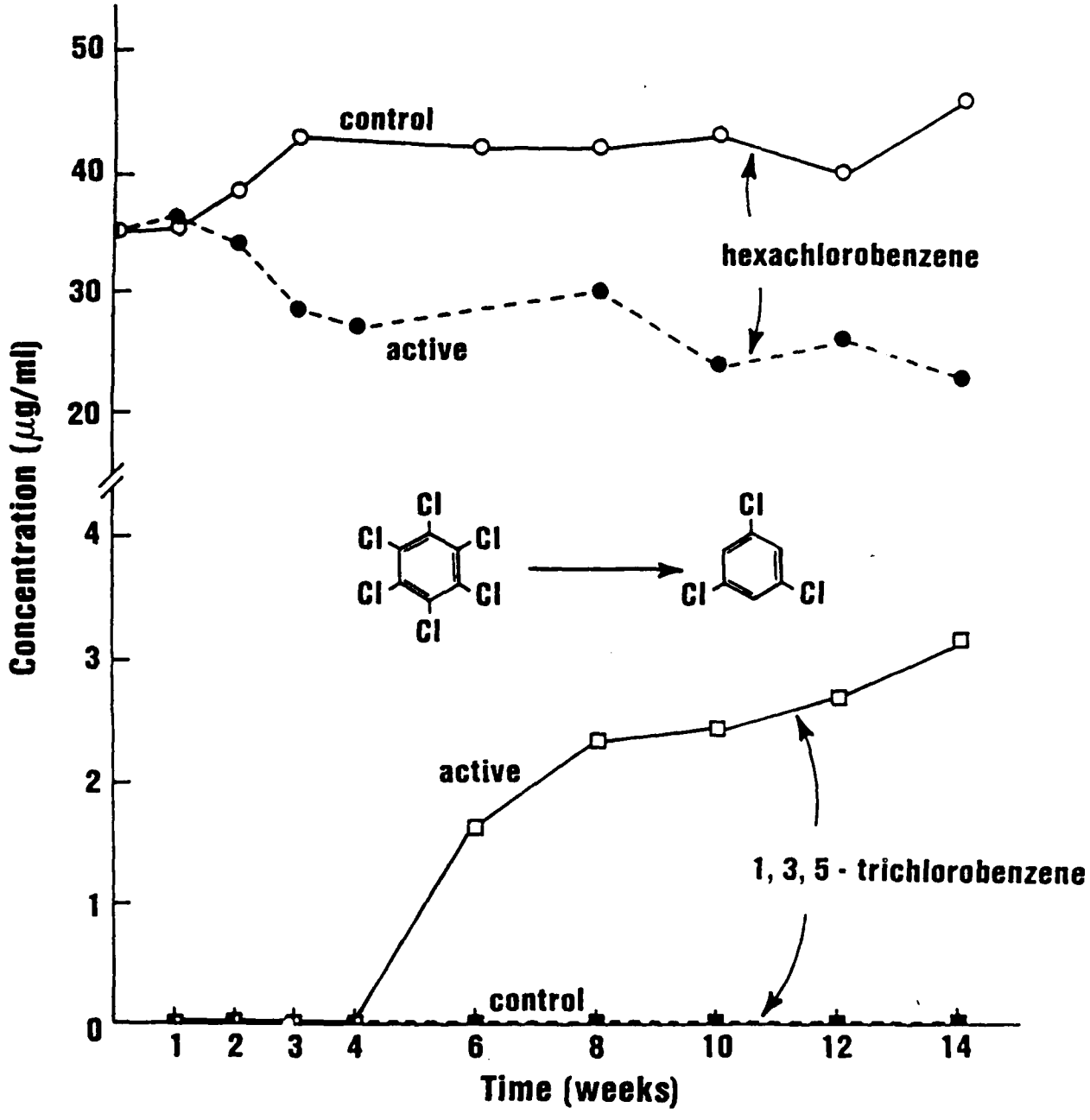

Figure 32. Dechlorination of Hexachlorobenzene in an Anaerobic Sludge Digester and Production of 1,3,5-Trichlorobenzene as the Major Product.

the added HCB (50 μg mL^{-1}) was removed over a period of 12-14 weeks. At its maximum accumulation (14 weeks) 1,3,5-TCB accounted for approximately 40 percent of the HCB disappearance. Penta- and tetrachlorobenzenes were not detected. The sludge that had been autoclaved showed no decrease in the HCB concentration. In the active samples, further dechlorination to give dichlorobenzene, chlorobenzenes, and benzene is possible; however, these compounds are easily lost by volatilization. In sterilized sludge inoculated (20 percent inoculum) with sludge previously exposed to HCB for 20 weeks, similar results were obtained except that both 1,2,3,5-tetrachlorobenzene (TTCB) and 1,3,5-TCB were observed. Sterilized sludge alone did not show the formation of either TTCB or TCB.

These results showed a significant biodegradation of HCB via reductive dechlorination under strict anaerobic conditions. Parallel studies using nonlabelled HCB (as above) and ^{36}Cl-HCB will be conducted in the future to confirm these results.

SECTION IX

STUDIES TO ASSESS WHETHER TETRACHLORODIBENZO-P-DIOXIN (TCDD) CAN BE REDUCTIVELY DECHLORINATED

A. RATIONALE AND APPROACH

One goal of our research is to evaluate whether anaerobic dechlorination of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) occurs using chlorine-36 labelled TCDD. If it occurs, we also sought to determine factors affecting its rate of dechlorination. We have conducted experiments using Titabawassee River MI sediment, fresh sludge, hexachlorobenzene (HCB) exposed sludge, sludge acclimated to each of the monochlorophenols separately and simultaneously, a p-Cl-phenol enrichment, an o + m + p-Cl-phenol enrichment, and an m-Cl-benzoate enrichment.

B. MATERIALS AND METHODS

1. Experimental Design

We added ^{36}Cl-TCDD to anaerobic sludge, sediment, or enrichment culture and sampling periodically for the presence of ^{36}Cl. Autoclaved and chloroform treated controls were used to determine if any nonbiologically mediated dechlorination occurs. Each treatment was replicated three times.

2. Sediment, Sludges, and Enrichment Cultures

Sediment was collected by Eckman dredge from the Titabawassee River below Midland, Michigan. The sediment was stored in full, tightly capped jars at 4°C for 2 days before experiments were initiated. Sewage sludge was collected from the sewage treatment plant at Jackson, Michigan. The experiment with the nonacclimated sludge was begun within 10 days of collection. Hexachlorobenzene (50 ppm) was added to obtain the HCB exposed sludge. This sludge had previously been shown to dechlorinate HCB. The chlorophenol acclimated sludges were derived from the Jackson sludge by feeding the appropriate chlorophenol(s) for up to 2 years. The chlorophenol concentration was assessed approximately weekly and chlorophenol was added as needed to maintain concentrations between 8 and 12 μg/mL. The p-Cl-phenol enrichment culture was obtained by diluting acclimated sludge (1:3) with anaerobic culture medium (Reference 3). A highly enriched culture capable of degrading all three monochlorophenols was obtained by mixing 100 mL of enrichments that had previously been diluted (1:3) two (o- and p-Cl-phenol enrichments) or three (m-Cl-phenol enrichment) times with 700 mL of anaerobic medium. The m-Cl-benzoate enrichment was selected over a 2 year period from Adrian, Michigan sewage sludge and is capable of utilizing m-Cl-benzoate as the sole carbon and energy source (Reference 1).

3. Experimental Conditions

Fifty milliliters of sludge or sediment or 100 mL of highly enriched cultures were added to 125 mL serum bottles (actual capacity 161 mL), using

anaerobic techniques. The bottles were sealed with thick rubber stoppers and aluminum crimps and wrapped in aluminum foil to exclude light. One hundred microliters (sludge, sediment, and p-Cl-phenol enrichment) or 200 µL (m-Cl-benzoate and o + m + p-Cl-phenol culture) of acetone solution of ^{36}Cl-TCDD (50 µg/mL) were added to each bottle under subdued light. Final TCDD concentration was 100 ppb. The ^{36}Cl-TCDD was synthesized by New England Nuclear, had a specific activity of 2.25 mCi/mmole, and was certified greater than 99 percent pure. Blanks for each treatment were prepared under identical conditions except for the addition of ^{36}Cl-TCDD. The bottles were then shaken for 30 minutes. Sludges and enrichments were incubated at 37°C, whereas sediments were incubated at 20°C. Incubations were static.

4. Assessment of Biological Activity

The biological activity of each treatment was assessed by measuring methane production (fresh sludge and sediment) or chlorinated substrate disappearance (m-Cl-benzoate and chlorophenol acclimated sludges and enrichments). Methane measurements were made using a Carle 8500 gas chromatograph with a 2 m Porapak® QS column and microthermister detector. Yeast extract (to 0.1 percent) and ethanol (to 1 ppm) were added to bottles when methane production ceased. To determine chlorophenol and chlorobenzoate concentrations, 1 mL samples were vortexed with 0.5 mL of acetonitrile before centrifugation (sludge and sediment samples only) and filtration through a 0.45 m membrane filter (Whatman type HVLP). Samples were analyzed by HPLC using a C-18 column with 30 percent acetonitrile and 70 percent of a 5 percent aqueous solution of acetic acid as the mobile phase. Absorbance was measured at 280 nm (chlorophenols) or 284 nm (chlorobenzoate). Chlorobenzoate concentrations were maintained between 8 and 12 ppm of each isomer, as appropriate, by adding the appropriate volume of a 6,000 µg/mL stock solution. The chlorobenzoate enrichment was fed 800 µM twice a week from an 80 mM stock solution.

5. Sampling Procedure for ^{36}Cl-

Treatments were sampled for measurement of ^{36}Cl- concentrations day 1, and 1, 4, 8, and 16 weeks after ^{36}Cl-TCDD addition.

Suitable methods were developed for separating ^{36}Cl- from sludge sediments and enrichments so that it could be counted by liquid scintillation independently of the parent substrate (^{36}Cl-TCDD). In the cases of sludges and sediments all or most TCDD partitions to the solids. Five mL samples were centrifuged for 5 minutes at 10,000 grams, and the supernatant filtered through glass fiber and 0.45 µm membrane filters. Two mL of the filtrate was counted in 15 mL of aqueous cocktail for 30 minutes. For the highly enriched cultures, 50 mg of Cl- saturated activated charcoal was thoroughly mixed with a 4 mL sample prior to the centrifugation and filtration steps. This removed any TCDD in solution. The Cl- saturated activated charcoal was prepared by mixing 1 gram of charcoal with 50 mL of 10 percent NaCl, filtering, and drying the charcoal overnight at 100°C. This prevented binding of the very low levels of ^{36}Cl- to be expected in the samples.

C. RESULTS AND DISCUSSION

1. Reliability and Sensitivity of Method

The procedures for separating Cl- and TCDD were evaluated with 4-TCDD because of its higher specific activity (126 mCi/mmole). When 0.4 μCi of ^{14}C-TCDD was added to 10 mL of sludge or sediment (102 ng/mL), only 23 ± 1 dpm (X ± s) per 2 mL sample remained in the filtrate. Because the ^{36}Cl-TCDD used was only 2.25 mCi/mmole, this amount would give only 0.3 to 0.5 dpm ^{36}Cl-TCDD per 2 mL sample after centrifugation and filtration. No significant counts were obtained when ^{36}Cl-TCDD was used in parallel experiments. Thus, centrifugation and filtration is sufficient to separate ^{36}Cl- and ^{36}Cl-TCDD in sludge and sediment systems because nearly all of the TCDD partitions to the solids.

The sensitivity and reliablity of the procedure were determined by adding various concentrations of Na^{36}Cl to sludge and sampling. The results indicated (Table 16) that the adopted procedure is more than adequate to detect 1 percent dechlorination of 100 ppb of our ^{36}Cl-TCDD. When as little as 8 dpm ^{36}Cl- was added per mL of sludge, sample counts were significantly above background levels. This represents 0.5 percent dechlorination. There is slight enrichment for Cl- in the sampling procedure because sludge contains a high proportion of solids and the Cl- partitions to the water. Disregarding such enrichment, 1 percent dechlorination of 100 ppb TCDD should give 30 dpm per sample. Because the radiopurity of the ^{36}Cl-TCDD is certified at 99 percent we can accept counts greater than 30 dpm/sample (above background) as evidence of dechlorination.

Centrifugation and filtration of the highly enriched cultures left about 95 dpm of ^{36}Cl-TCDD per 2 mL sample. When 50 mg of activated charcoal was mixed with the 4 mL samples prior to centrifugation, no radioactivity could be detected in the filtrate.

However, the use of activated charcoal also removed approximately 50 percent of the ^{36}Cl added at concentrations of 8 to 88 dpm/mL (Table 17). Because this meant a serious loss in the sensitivity of our assay, we tried saturating the charcoal with NaCl prior to use. This proved just as effective at removing TCDD, but did not remove the Cl-.

2. Dechlorination Experiments

Three criteria must be met to demonstrate biological anaerobic dechlorination of TCDD. First, sample radioactivity must be greater than can be accouted for by dechlorination of possible radioimpurities (< 1 percent). This means the samples must contain more than 30 dpm. Second, the experimental treatments must give higher counts than the controls. Third, there should be a temporal increase in counts due to ^{36}Cl-.

Thus far, these criteria have not been met in any of the experiments. Counts between 10 and 20 dpm per experimental sample are often obtained on day one, but these are no greater than counts for the controls and do not increase

TABLE 16. SENSITIVITY AND PRECISION OF $^{36}Cl^-$ CONCENTRATION MEASUREMENTS IN SEWAGE SLUDGE.

$^{36}Cl^-$ concentration (dpm)*	% Dechlorination represented	$^{36}Cl^-$ recovered dpm*	2S
16	0.5	25.9	3.4
80	2.7	96.0	4.5
160	5.4	175.6	5.6

*Disintegrations per minute for a 2 mL sample.

with time. All experiments have been sampled for 16 weeks except the highly enriched o + m + p-Cl-phenol and m-Cl-benzoate enrichments which have been sampled for 1 and 12 weeks, respectively.

3. Biological Activity

On all experiments except for the m-Cl-benzoate and o + m + p-Cl-phenol enrichments the biological activity decreased drastically after about 4 weeks. This is one possible reason for the lack of TCDD dechlorination. Biological activity was assessed by measuring methane production or chlorinated substrate disappearance.

An attempt was made to revive biological activity by feeding the cultures. The addition of yeast extract (to 0.1 percent) and ethanol (to 1 ppm) to the fresh sludge. HCB-exposed sludge, and Titabawassee River sediment caused methanogenesis to resume, and these cultures are now fed these amounts monthly. The same amount of yeast extract was also fed to all inactive chlorophenol cultures. Chlorophenol dechlorination resumed in all three replicates for the o-Cl-phenol acclimated sludge, and in some replicates for the m-Cl-phenol and o + m + p-Cl-phenol acclimated sludges and for the p-Cl-phenol enrichment, but not for the p-Cl-phenol acclimated sludge.

Dechlorination of TCDD may also be limited by lack of bioavailability. The TCDD binds to the solids in the system and may not be taken up readily by the microorganisms. Thus, there are two basic problems to overcome in future studies. These are maintaining biological activity during long term incubations and increasing TCDD bioavailability. It will be necessary to try several different substrates to feed the cultures. The substrate used may influence the results, but because we have no information on the exact mechanism of anaerobic dechlorination of aromatic compounds

TABLE 17. SENSITIVITY AND PRECISION OF $^{36}Cl^-$ CONCENTRATION MEASUREMENT IN HIGHLY ENRICHED CULTURES USING ACTIVATED CHARCOAL AND NaCl SATURATED ACTIVATED CHARCOAL.

$^{36}Cl^-$ Concentration (dpm*)	% Dechlorination represented	$^{36}Cl^-$ recovered (dpm*)	
		Charcoal treatment	NaCl charcoal treatment
16	0.3	8 ± 7**	20 ± 7
40	0.7	20 ± 7	44 ± 8
84	1.4	44 ± 9	90 ± 9
176	2.9	84 ± 8	170 ± 10

*Disintegrations per minute for a 2 mL sample.

**X ± 2S

do not know which substrates are best to use. Surfactants should increase TCDD bioavailability by decreasing its binding to organic solids and making it more soluble. Both anionic and nonionic surfactants have been shown to increase TCDD bioavailability in soil (Reference 111).

SECTION X

REDUCTIVE DECHLORINATION OF TETRACHLOROETHYLENE AND TRICHLOROETHYLENE BY ACETOCLASTIC METHANOGENS

A. RATIONALE AND APPROACH

Some of the most frequently detected groundwater contaminants in the United States are synthetic organic solvent chemicals such as tetrachloroethylene, trichloroethylene, 1,1,1-trichloroethane, chloroform, and methylene chloride. The Air Force has these contaminants in over 1000 sites. Most of these solvent chemicals in groundwater have likely occurred from their widespread use as degreasing solvents and due to improper disposal methods.

Bower and McCarty, 1983 (Reference 11) showed that under methanogenic conditions, tetrachloroethylene (PCE), and trichloroethylene (TCE) were biodegraded, and the addition of acetate was necessary for this reaction to occur. Under these conditions, PCE was dechlorinated to TCE and then to vinyl chloride (VC) (Reference 13). Microscopic observations of their acetate-enrichments indicated the presence of large numbers of Methanothrix like (Reference 11) and Methanosarcina like organisms. However, no attempts were made to isolate the causative organism(s).

The primary goal of this section was to find out whether acetate-utilizing methanogens were active in the biotransformation of PCE and TCE.

B. MATERIALS AND METHODS

1. Growth Media and Conditions

The PREM-medium (Reference 86) was used for growing Methanosarcina sp., Methanothrix sp., and Methanosarcina mazei (Ms. mazei). The maintenance medium (Reference 90) was employed for the growth of Methanosarcina acetivorans (Ms. acetivorans).

Sterile media were prepared under N_2 or N_2-CO_2 (80:20) atmosphere by using a modified Hungate technique (Reference 91). All incubations were carried out in a 160 mL serum bottles that contained 50 mL of growth medium. An aliquot of 5 µL of PCE from the methanol stock (10 mg mL^{-1} PCE in methanol) was added to each serum bottle via a 1 µL Hamilton syringe to a final concentration of 1 ppm, whereas the serum bottle was continuously sparged with O_2 free N_2 or N_2-CO_2 gas. Each serum bottle was inoculated with an actively growing methanogen (2 percent v/v inoculum size). The serum bottles were then sealed with 20 mm Teflon®-lined rubber septa and aluminium crimp caps (Supelco, Inc.)

2. Experimental Procedure

The batch tranformations were carried out by inoculating several serum bottles containing PCE and incubating all at one time. Periodically, duplicate

bottles of inoculated (active) and uninoculated (control) bottlas were sacrificed for the extraction and analysis.

For extraction, 10 mL of toluene was added to each serum bottle via a glass syringe and needle. The bottle was then agitated thoroughly on a reciprocating shaker for 30 minutes at room temperature. The bottles were then left undisturbed for ~10 minutes to obtain a clear separation of organic and aqueous phases. The organic layer was carefully transferred with a pasteur pipette and further centrifuged. An appropriately diluted sample was used for the analysis.

3. Analytical Methods

The concentration of the test compound and product(s) formed were monitored with a 5890A HP gas chromatograph, equipped with a ^{63}Ni detector and a stainless steel column (8 feet x 1.8 inches OD) packed with 60/80 Carbopack B/1 percent sp-1000 (Supelco, Inc., Belforte PA). N_2 was used as a carrier gas at a flow rate of 40 $mL \cdot mL^{-1}$. Identification and quantitation of compounds was achieved by comparison with standards.

4. Chemicals

Tetrachloroethylene (99 percent) and trichloroethylene (99 percent) were obtained from Aldrich Chemical Co., Inc., Milwaukee WI. All other chemicals used were of reagent grade.

C. RESULTS AND DISCUSSION

Figures 33, 34, and 35 represent results from batch, anaerobic incubation of PCE in inoculated samples and autoclaved controls. Each serum bottle was amended initially with 300 n·moles of PCE. Figure 33, shows the change in concentration of PCE over 12 weeks from active Methanothrix culture and sterile control bottles. Reductive dechlorination took place within 3 weeks with an initial lag of 2 weeks. Similar observations were made on PCE dechlorination by Methanosarcina sp. grown on methanol or acetate as a primary carbon source in (Figures 34 and 35). In both cases (Figures 34 and 35) the dechlorination of PCE by Methanosaroina sp. occurred without a lag, and the rates of TCE formation were markedly higher in cultures grown on methanol than in acetate-grown cells. About 14 nmoles of TCE was detected in methanol grown cells (Figure 35) compared to 8 nmoles produced by acetate grown Methanosarcina sp. cells at the end of experiment. The cause for this substrate effect on dechlorination of PCE is not known. However, it was known that acetate yields low energy (-7.4 kcal/mol) during methanogenesis compared to methanol (.75 kcal/mol). Besides, only 2 reducing equivalents are generated by acetate metabolism compared to 6 reducing equivalents by methanol (Reference 92). Thus perhaps the excess electrons generated during methanol metabolism by Methanosarcina are causing the higher rates of PCE dechlorination by cultures grown on methanol.

The results presented in Figures 33, 34, and 35 clearly show the biological formation of TCE from PCE. In all cases the formation of TCE was

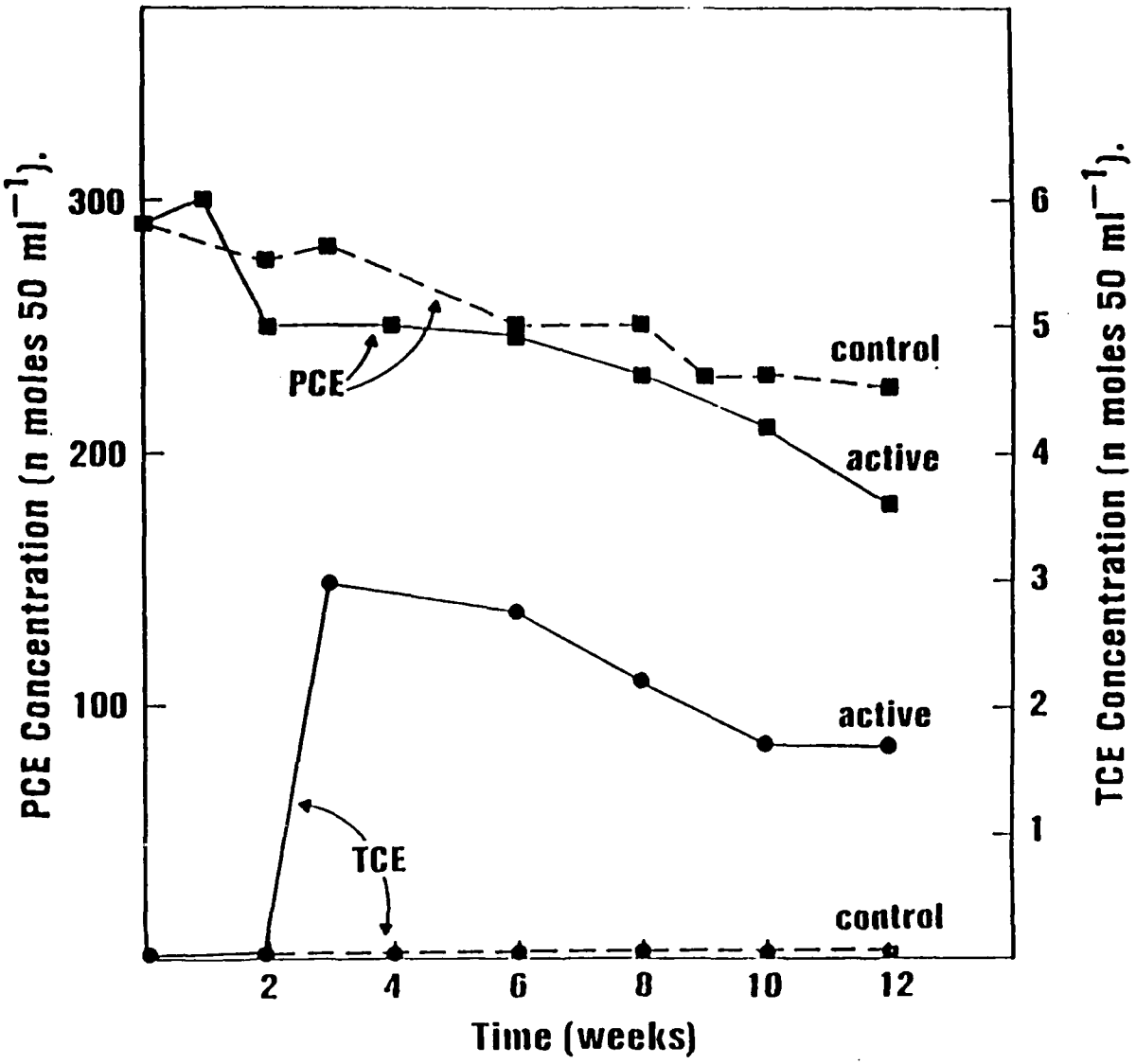

Figure 33. Biotransformation of PCE to TCE by *Methanothrix* Culture Grown on Acetate (50 mM).

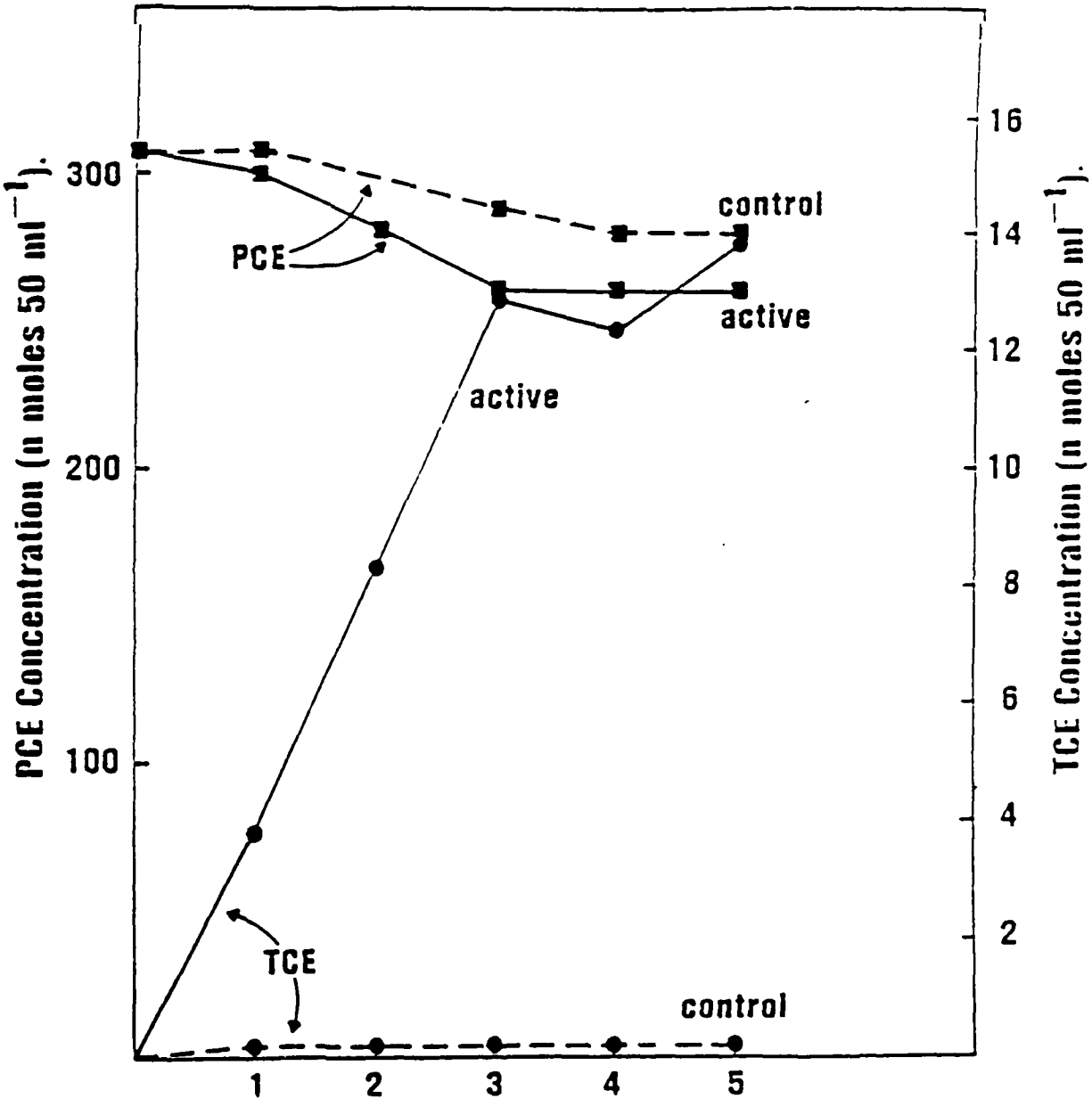

Figure 34 Biotransformation of PCE to TCE by Pure Culture of *Methanosarcina* sp. Grown on Methanol (25 mM).

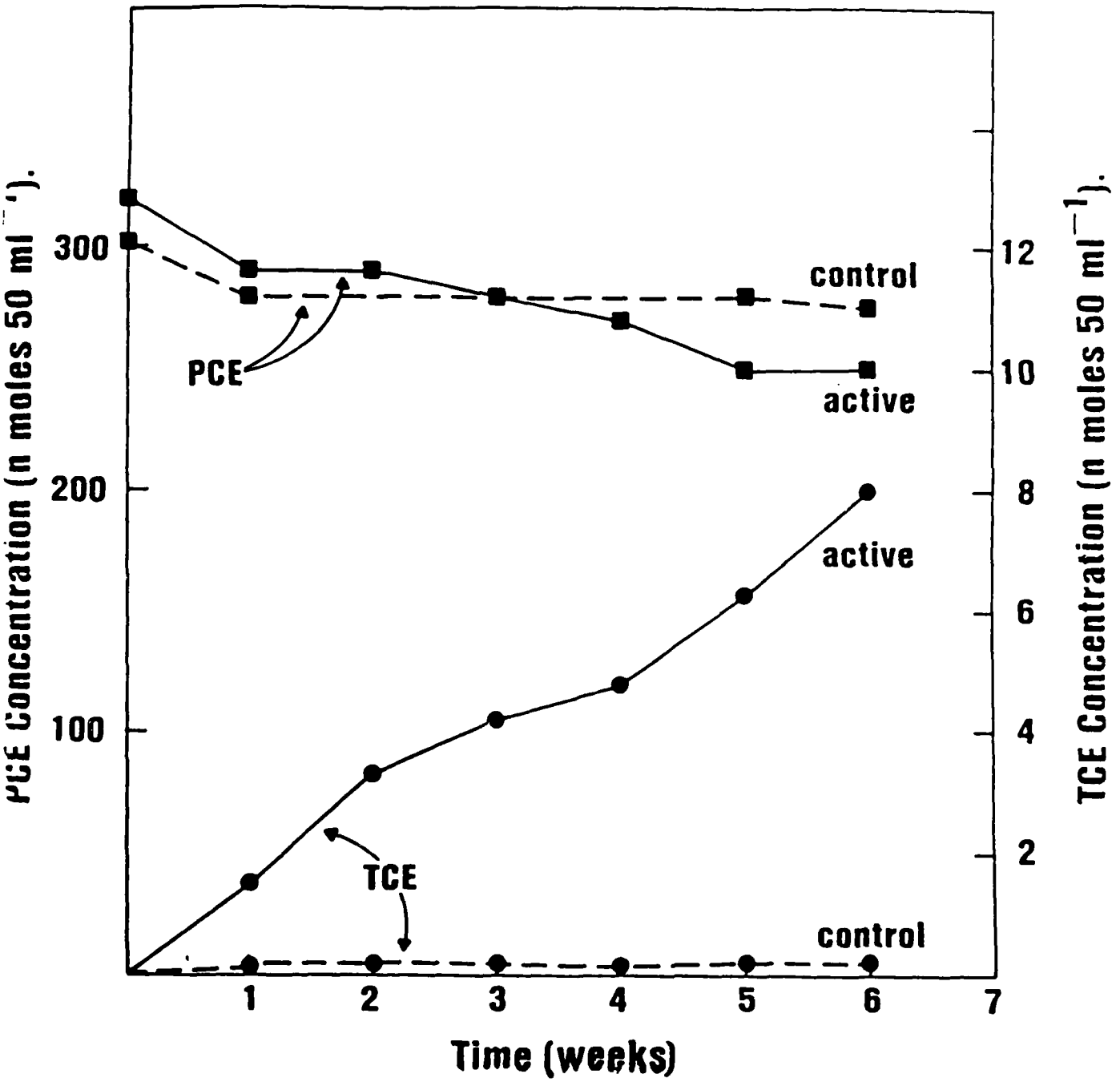

Figure 35. Biotransformation of PCE to TCE by pure culture of *Methanosarcina* sp. grown on acetate (50 mM).

not observed in control bottles. The slow decline in the concentration of PCE from control bottles indicated that a portion of the added PCE was partitioned to the gas phase. However, a significant amount of TCE was detected only in the seeded serum bottles, indicating a direct role of methanogens in the biotransformation of PCE. Our previous studies on the degradation of PCE and TCE by pregrown methanogic cells (data not shown) showed no such biotransformations, strongly suggesting that dechlorination of PCE was by actively growing methanogens and ruling out any possible abiotic transformation by cell components of the lysed cells. These studies are in accordance with the observations made by Bouwer and McCarty (References 11, 92) which strongly suggest the role of the acetate-cleaving organism in biotransformation of PCE. Recent studies by Vogel and McCarty (Reference 13) further demonstrate the anaerobic degradation of PCE in methanogenic upflow columns with acetate as the sole carbon and energy source.

Results from Table 18 summarize information on the formation of TCE by the pure cultures of acetoclastic methanogena. In general, both Methanosarcina sp. and Methanosaroina mazei were good biotransformers of PCE compared to Methanothrix sp. Extremely low amounts of PCE was converted to TCE by Methanosarcina acetivorans.

In summary, our data provides evidence for a possible involvement of acetoclastic methanogens in the biotransformation of PCE to TCE by a reductive dechlorination mechanism. The rate of transformation was correlated with the rate of CH_4 formation and varied with the substrate used for methane production. However, the present study does not explain the mechanism of biotransformation nor does it rule out a similar possible transformation by nonmethanogenic anaerobes.

TABLE 18. BIOTRANSFORMATION OF PCE TO TCE BY ACETOCLASTIC METHANOGENS.

Methanogen	Time (week)	Conc. of TCE formed (in active bottle)	(in control bottle)
		----------nmoles----------	
Methanosarcina sp.*	3	12.95	ND**
Methanosarcina mazei	3	13.21	ND
Methanosarcina acetivorans	3	1.15	ND
Methanothrix sp.*	3	3.0	ND

*Methanosarcina sp. and Methanothrix sp. were isolated from a chlorophenol degrading enrichment. Acetate (50 mM) and methanol (25 mM) served as a primary carbon source for Methanothrix sp. and for all Methanosarcina, respectively.

**ND, not detected.

SECTION XI

CONCLUSIONS

1. Highly chlorinated compounds, represented by hexachlorobenzene and pentachlorophenol, are dehalogenated by anaerobic microbial communities. The dechlorination is more rapid with the hexa- and penta-substituted compounds than it is with the di- and tri-halogenated compounds.

2. One dechlorinating isolate was characterized and found to have a unique physiology. It readily used only 1 of over 50 carbon compounds as growth substrate, consumed H_2 and reduced thiosulfate and sulfite but not sulfate. It is thought to be a new type of sulfidogen. Excess H_2 and sulfur anions inhibited deohlorination.

3. An anaerobic upflow reactor was established on chlorophenol as the only substrate. This dehalogenating activity in the reactor could be maintained for over 1 year but the loading capacity and conversion rates were limited.

4. Two previously unknown aryl-dechlorinating activities were discovered. These were for hexachlorobenzene which was converted to at least trichlorobenzene and 4-chlororesorcinol which converted to resorcinol. The latter activity could be greatly stimulated by certain complex carbon sources such as trypticase and rumen fluid.

5. At the end of the technical effort we had not observed dechlorination of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or trichloroethylene. We developed a sensitive method to study TCDD dechlorination using 36 labelled substrate and measuring $^{36}Cl^-$ release. Tetrachloroethylene (PCE) conversion to TCE was observed by acetoclastic methanogens present in many anaerobic communities.

6. Reductive dechlorination of many aromatic compounds by anaerobic microbial communities does occur. The reaction rates are relatively slow, especially for nonpolar compounds. Nonetheless, this proces appears to be one of the very few biological mechanisms to degrade highly chlorinated aromatic compounds.

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